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DOUBLE FERTILIZATION AND DEVELOPMENT OF THE SEED IN ANGIOSPERMS[1]

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(WITH NINE FIGURES)

Introduction

Ever since its discovery by NAVASHIN (**19**) and GUIGNARD (**13**) some 40 years ago, double fertilization has remained a puzzling feature of reproduction in the flowering plants. Fertilization of the egg, giving rise to the embryo, is essentially like that in other plants and in animals, both in its morphological and physiological aspects. But conjugation of the second male nucleus with a polar-fusion nucleus to form the primary endosperm nucleus is a phenomenon peculiar to the angiosperms. Three nuclei enter into the structure of the primary endosperm nucleus: a micropylar polar, an antipodal polar, and a sperm nucleus. Participation of a male nucleus in the triple fusion has given rise to much discussion regarding the morphological nature of the resulting endosperm tissue; and the physiological significance of the event has remained almost wholly obscure.

Conjugation of the second male nucleus with the polar-fusion nucleus is interpreted in the present paper as a mechanism by which the developmental advantages of heterosis become available to the endosperm. New evidence supporting the view that the endosperm plays a leading part in the early development of the seed is brought forward; and critical data are presented establishing the fact that, under the particular conditions encountered in flowering plants, the added stimulus to growth associated with hybridity in this tissue may be essential to seed formation.

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Although triploidy, with occasional exceptions, is a salient feature of the endosperm of angiosperms, and the presence of a double complement of maternal chromosomes in the tissue may be important in the intimate reciprocal relationships between endosperm, embryo, and mother plant, these circumstances are outside the boundary of the present discussion. The role of the male nucleus entering into the triple fusion may also be considered apart from them.

COULTER (6), contending that the endosperm is a gametophytic structure, regarded the presence of the male nucleus in triple fusion as of subsidiary importance. This view, however, cannot be maintained in face of the present evidence. Whether the endosperm of angiosperms is more properly regarded as gametophytic than as sporophytic tissue is a morphological problem, and as such does not concern us here. The endosperm in gymnosperms is derived by continuous cell division from the haploid megaspore and is obviously gametophytic. From a functional point of view, interpolation in the angiosperms of a fusion involving a sperm nucleus is a fundamentally significant fact which cannot be ignored in any morphological interpretation. The endosperm is an undifferentiated tissue in gymnosperms and angiosperms alike, and its role is essentially nutritive in both groups. The important thing to recognize is that while a common end is served, the circumstances under which the endosperm functions in the two cases are unlike. The female gametophyte in angiosperms is reduced to its lowest terms, and it is in the light of this fact that the novel mechanism of endosperm formation in flowering plants becomes intelligible.

The distinction which STRASBURGER (22) sought to draw between generative and vegetative fertilization as applying to conjugation of the sperm nuclei with the egg and polar-fusion nuclei, respectively, breaks down in the face of the evidence regarding xenia, as COULTER (6) and EAST (10) have pointed out. Both fertilizations are generative in the sense that the hereditary materials are transmitted in a definite and regular manner. SARGANT (20) has contended that the endosperm remains a formless and frequently short-lived tissue, instead of becoming organized as an embryo, because a vegetative nucleus enters into the fusion which otherwise might be considered sexual. This view, which implies that the endosperm of angiosperms is a degenerate embryo, runs counter to considerable evidence indicating that the endosperm acts as an auxiliary rather than as a competitive factor in embryo development.

The writers (2) have found recently that the low production of seed obtained following self-pollination of *Medicago sativa* is mainly due to two, probably distinct, factors: partial self-incompatibility and high incidence of collapse of fertile ovules in early stages of development. The frequency of fertilization was increased from 14.6 to 66.2 per cent on outcrossing to unrelated plants, and the proportion of fertile ovules collapsing during the first 144 hours after pollination

was reduced from 34.4 to 7.1 per cent. The partial self-incompatibility is not of interest in this connection except as it indicates that alfalfa reproduces largely through cross-pollination. The fact that the rate of collapse of fertile ovules is about five times as high after self-fertilization as after outcrossing is of particular significance. A study of the factors underlying the difference in survival of the two classes of seeds sheds new light on the role of double fertilization in the development of the seed and provides a factual basis for interpreting this hitherto obscure phenomenon.

Material and methods

Since the material used and the general methods followed have already been described (5), they will be mentioned only briefly here. Seven alfalfa plants, growing under greenhouse conditions favorable for seed production, were used. The two classes of matings, self-pollination and cross-pollination to unrelated individuals within the group, were made on castrated flowers in different inflorescences under strictly comparable conditions. Pistils were collected at 30, 48, 72, 96, 120, and 144 hours after pollination, and fixed and imbedded in paraffin. After sectioning and staining, the data taken included: fertility of the ovules, frequency of fertile ovules collapsing, number of cells in the proembryo and embryo, and number of nuclei in the endosperm. Alfalfa is well suited to observations of this kind, the ten to twelve ovules per ovary being arranged alternately along the ventral suture, approximately in a straight line. The plane of sectioning can therefore be closely controlled and each ovule critically examined. The number of fertile ovules observed was 433 in the selfed series and 1682 in the crossed series, the two classes being distributed over the seven plants in approximately the same proportion.

When it became evident that the rate of collapse of fertile ovules was markedly different following self- and cross-fertilization, a detailed study of the histological changes associated with failure was undertaken on the material collected from 48 to 144 hours. The changes were found to involve the megagametophyte and the maternal tissue immediately adjacent to it. Each ovule behaved independently at this stage. Except for a slightly greater tendency to persist in the apical region, survival was likewise independent of both position and number of fertile ovules.

It should be emphasized perhaps that the data are based upon more or less direct responses of the ovule to self- and cross-fertilization. The immediate effects of fertilization are local, but they quickly spread to the tissues destined to form the fruit. MURNEEK (18) has shown that as the fruit is stimulated to active growth, profound changes occur also in the general metabolism of the plant. As time elapses beyond fertilization, various secondary changes in the seed may be expected therefore, which add greatly to the difficulties of analysis. The early

post-fertilization stages are of critical importance for survival of the alfalfa seed, and this fact makes this plant favorable material for studying the primary consequences of double fertilization.

Experimental results

Collapse of fertile ovules

The number of collapsing fertile ovules following self- and cross-fertilization is shown in table 1 and figure 1. Fertilization in alfalfa usually occurs within 30 hours after pollination, and although the frequency is much lower, is only slightly

TABLE 1

FREQUENCY OF FERTILE OVULES COLLAPSING IN SEVEN ALFALFA PLANTS FOLLOWING SELF- AND CROSS-FERTILIZATION. DATA BASED ON COLLECTIONS AT 72, 96, 120 AND 144 HOURS AFTER POLLINATION (AFTER COOPER AND BRINK, 5)

Self-fertilization				Cross-fertilization			
Plant selfed	No. of fertile ovules		Percentage collapsing	Plants crossed	No. of fertile ovules		Percentage collapsing
	Total	Collapsing			Total	Collapsing	
A	37	9	24.3	A×B	187	13	7.0
B	37	19	51.4	B×C	110	5	4.5
C	20	7	35.0	C×D	171	13	7.6
D	17	7	41.2	D×E	171	16	9.4
E	39	8	20.5	E×A	146	9	6.2
F	109	39	35.8	F×G	228	14	6.1
G	55	19	34.5	G×F	198	16	8.1
Total	314	108	34.4	Total	1211	86	7.1

delayed after selfing as compared with crossing. Observations at 48 hours after pollination afford little evidence of ovule degeneration, but at 72 hours the collapse of fertile ovules is common. From previous data (5) on seed production on these plants, it may be estimated that about 20 per cent of the reduction in functioning ovules following cross-fertilization and about 70 per cent following self-fertilization occurring between fertilization and maturity of the seed takes place in the approximately 100-hour period on which table 1 is based. Collapse is not therefore limited to the relatively brief interval during which the samples were taken; but it is evident that the period is critical for seed survival, particularly after self-pollination.

In each of the seven plants the percentage of fertile ovules collapsing is much higher following self-pollination than after crossing, the respective mean values for the two groups being 34.4 and 7.1. Since the matings were made concurrently

on the same individuals, the disparity in survival evidently is attributable to the difference in genetic composition of the embryos and endosperms within the two classes of seeds.

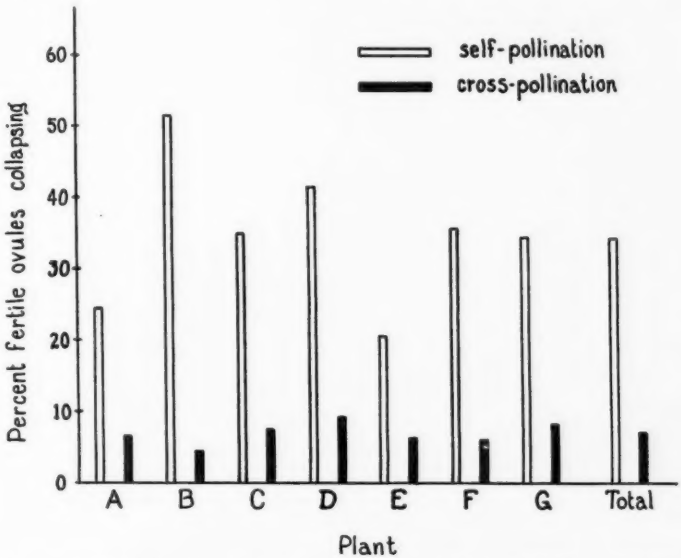


FIG. 1.—Frequency of fertile alfalfa ovules collapsing in pistils collected at 72, 96, 120, and 144 hours following self- and cross-pollination (after COOPER and BRINK, 5).

HISTOLOGY OF FERTILE OVULE COLLAPSE

Just prior to and at the time of fertilization in alfalfa there is little, if any, meristematic activity in any portion of the ovule, the nuclei being in a resting state.

When fertilization does not occur there is no further development of the ovule. The latter retains its shape and appears potentially functional for another 24 hours, then degenerates. The first evidence of breakdown is collapse of the megagametophyte in the region of the bend of the ovule. The collapse is accompanied by shrinkage of the ovule in that region. Disintegration of the megagametophyte and ovule continues, so that the infertile structure is much shrunken at 96 and 120 hours after pollination. Following fertilization, on the other hand, the entire ovule is immediately stimulated to develop. Active nuclear and cell division is initiated in the integuments and funiculus. The few remaining cells of the nucellus at the chalazal end of the megagametophyte do not divide further. They persist for a time, evidence of them being found in some seeds up to 144 hours after pollination. Ultimately these cells disintegrate completely and the endosperm comes to lie in direct contact with the inner integument.

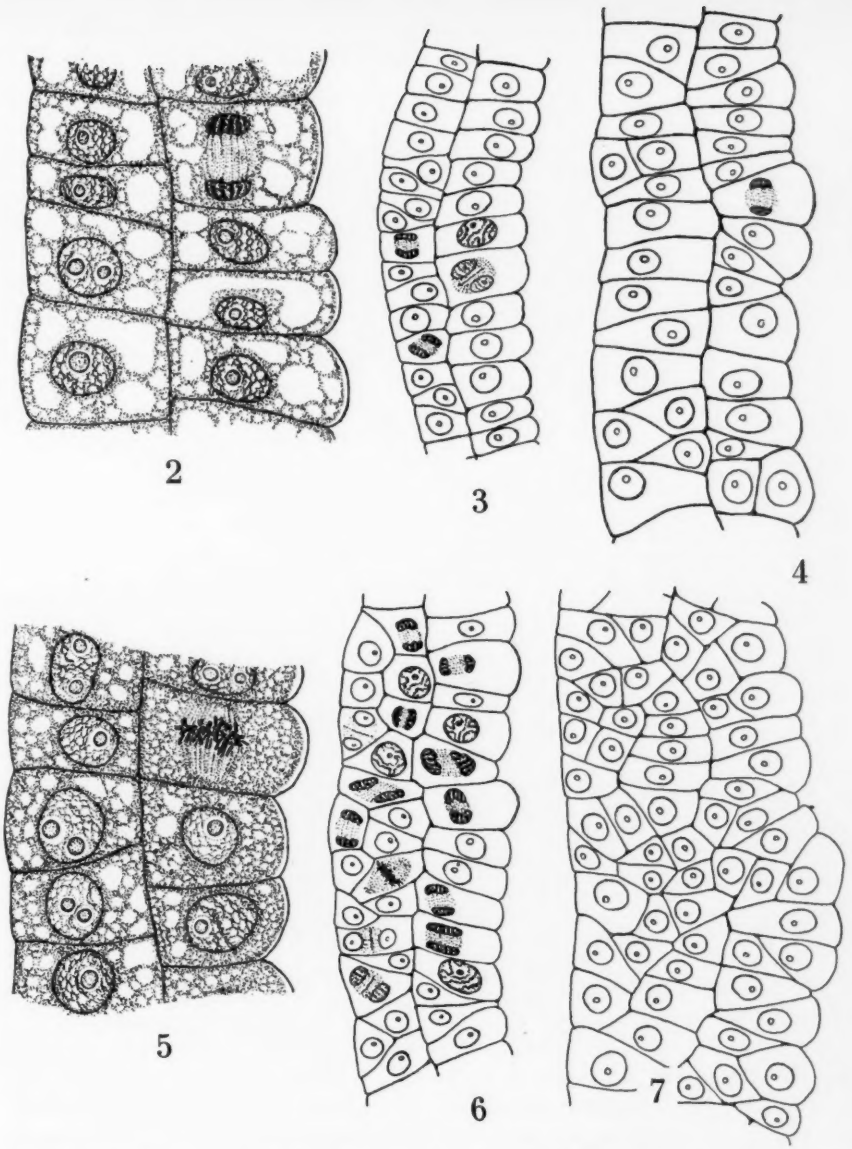
The primary endosperm nucleus divides shortly after fertilization, the position of the spindle being oriented so that each arm of the elongated V-shaped mother cell receives one of the daughter nuclei. Further divisions occur, the endosperm becoming multinucleate. Later, 6–7 days after pollination, cell formation is initiated in the region of the embryo and advances toward the chalaza, so that in a short time the endosperm is completely cellular. As long as the endosperm is in a free nucleate condition the number of nuclei in each arm is approximately equal. At these early stages of development the nuclei are located in the peripheral layer of cytoplasm which surrounds the elongated central vacuole.

Nuclear and cell divisions continue in the outer integument and funiculus, so that these structures become both longer and larger as development proceeds. The cells of the inner integument become highly vacuolate shortly after fertilization has taken place and have the appearance of tapetal cells. They likewise continue to divide, but the plane of division is such that the integument increases in length but remains two layers of cells in thickness. Occasionally a cell may divide in the opposite plane, and at that point the integument will be three cells thick.

The cells of the proembryo at the various stages of development are densely cytoplasmic and stain heavily, especially those near the apex. After formation of the true embryo, the basal cells of the suspensor become more vacuolate and stain lightly, whereas those of the embryo remain dense and stain heavily.

The first evidence of collapse of a fertile ovule may be seen approximately 48 hours after pollination. The cells of the inner integument in the chalazal arm on the side adjacent to the funiculus become more or less densely cytoplasmic and assume a meristematic appearance (fig. 5), instead of remaining tapetum-like as in the normally developing seed (fig. 2). Active nuclear and cell division is initiated in this region (fig. 6). The mitotic figures are oriented at various angles and cell plate formations are in diverse planes, so that a number of layers of cells result (fig. 7). In a normally developing seed the plane of cell division in this region of the inner integument is usually longitudinal (fig. 3), and for the most part two layers of cells are formed (fig. 4).

During the course of this localized meristematic activity of the inner integument, the few remaining nucellar cells increase in size and stain heavily. The endosperm collapses in a region just posterior to the bend of the seed and shrinks away from the actively proliferating portion of the integument. The collapse proceeds toward both ends of the seed. The chalazal end of the endosperm collapses first and may be completely disorganized while the micropylar end may still be more or less turgid. The cells of the embryo in such a seed become highly vacuolate and starved in appearance. Shortly thereafter the remaining endosperm and the embryo become disorganized. Although the fertile ovule ceases further development, it retains its shape more or less for some time before collapsing. Such collapsing seeds are common after selfing and of infrequent occurrence following crossing.



FIGS. 2–7.—Fig. 2, portion of inner integument from chalazal region of fertile ovule adjacent to funiculus, showing highly vacuolate condition of cells 48 hours following cross-pollination. Fig. 3, same 72 hours after cross-pollination. Fig. 4, 96 hours after cross-pollination; inner integument remains two layers of cells in thickness. Fig. 5, portion of inner integument from chalazal region of fertile ovule adjacent to funiculus, showing dense cytoplasm of cells 48 hours after self-pollination. Fig. 6, same 72 hours after self-pollination, showing large number of cells in stages of division. Fig. 7, 96 hours after self-pollination; integument now composed of several layers of cells.

Reserve foods in ovule

The mature ovule of alfalfa is almost completely lacking in reserve foods. No storage products are to be seen in the main body of the ovule, and the small amount of starch within the megagametophyte quickly disappears after fertilization. Henceforth the young seed is dependent upon nutrients moved in from adjacent parts of the plant.

Development of the ovule and young seed at a nutrient level so low that little storage occurs appears to be significant in relation to survival. Failure of the fertile ovule to continue development frequently may be due to starvation as a result either of shortage of supply or unbalanced distribution of the nutrients between the tissues inside and outside the endosperm.

Examination was made of the mature ovules of thirty-six additional species of angiosperms, representing fifteen families, to determine whether the paucity of stored foods in the ovule was characteristic of the flowering plants. The observations, summarized in table 2, show that the condition found in alfalfa is more or less typical of the whole group.

Some starch was found in the ovules of twenty-one of the thirty-six species. It is usually limited to the egg and the endosperm mother cell of the megagametophyte. The amount in these cells varies from very little in *Ulmus*, *Lupulinus*, *Lycopersicum*, and some others to an abundance in *Zea*, *Melilotus*, *Phryma*, and certain additional species. Reserve foods were not found in the synergids; and in only one species, *Amphicarpa*, was starch detected in the antipodals.

Storage was observed in other parts of the ovule in only three species. The nucellus and integuments contained starch in *Trigonella* sp., abundant food was present in the nucellus in *Galega officinalis*, and in *Phryma leptostachya* a limited amount of storage material was found in the outer integument.

Post-fertilization stages of seventeen of the twenty-one species wherein some food materials are stored in the ovule showed that such materials are usually consumed by the time a 2-celled proembryo is present; in only one species, *Galega officinalis*, was there evidence of stored foods beyond the 4-celled proembryo stage. Some starch was present in the nucellus of ovules of *Galega* containing embryos consisting of four cells plus suspensor. The starch in the megagametophyte was completely consumed at the 2-celled proembryo stage.

Generally speaking, therefore, the mature ovule of angiosperms is characterized by little or no stored foods. The limited nutrient reserves occurring in most species usually do not suffice beyond early post-fertilization development. Dahlgren (9) found that storage of starch in the megagametophyte seems to attain its maximum immediately before fertilization. The grains disappear rapidly thereafter.

TABLE 2

RELATIVE ABUNDANCE AND DISTRIBUTION OF STORED FOODS IN MATURE OVULES OF 37 SPECIES OF ANGIOSPERMS

SPECIES	MEGAGAMETOPHYTE				OVULE		
	EGG	ENDOSPERM MOTHER CELL	SYNERGIDS	ANTIPODALS	NUCELLUS	INNER INTEGUMENT	OUTER INTEGUMENT
Gramineae							
Euchlaena mexicana	+++*	+++	–	–	–	–	–
Zea mays	+++	+++	–	–	–	–	–
E. mexicana × Z. mays	+++	+++	–	–	–	–	–
Commelinaceae							
Commelina communis	–	–	–	–	–	–	–
Liliaceae							
Erythronium americanum	–	–	–	–	–	–	–
Lilium martagon	–	–	–	–	–	–	–
L. regalis	–	–	–	–	–	–	–
L. henryi	–	–	–	–	–	–	–
Urticaceae							
Ulmus americana	+	+	–	–	–	–	–
Amarantaceae							
Amaranthus graecizans	–	–	–	–	–	–	–
A. retroflexus	–	–	–	–	–	–	–
Portulacaceae							
Portulaca oleracea	–	–	–	–	–	–	–
Cruciferae							
Brassica oleracea	++	++	–	–	–	–	–
Leguminosae							
Melilotus albus	+++	+++	–	–	–	–	–
Medicago sativa	++	++	–	–	–	–	–
Medicago sativa (4*n*)	+++	+++	–	–	–	–	–
Medicago lupulina	++	++	–	–	–	–	–
Medicago glutinosa	++	++	–	–	–	–	–
Lupulinus polyphyllous	+	+	–	–	–	–	–
Desmodium grandiflorum	–	–	–	–	–	–	–
Lathyrus latifolius	–	–	–	–	–	–	–
Amphicarpa monoica	++	++	–	++	–	–	–
Galega officinalis	+++	+++	–	–	+++	–	–
Trigonella sp.	Oil	Oil	–	–	++	++	++
Pisum sativum	–	–	–	–	–	–	–
Phaseolus vulgaris	–	–	–	–	–	–	–
Malvaceae							
Abutilon theophrasti	–	–	–	–	–	–	–
Solanaceae							
Nicotiana rustica	++	++	–	–	–	.....	–
Lycopersicum esculentum	+	+	–	–	–	.....	–

* +++ = Abundant.
++ = Medium.
+ = Little.
– = None.

TABLE 2—*Continued*

Species	Megagametophyte				Ovule		
	Egg	Endosperm mother cell	Synergids	Antipodals	Nucellus	Inner integument	Outer integument
Scrophulariaceae							
Chelone glabra	+	+	−	−	−	−	−
Phrymaceae							
Phryma leptostachya	+++	+++	−	−	−	+	−
Dipsacaceae							
Scabiosa japonica	++	++	−	−	−	−	−
Scabiosa atropurpurea	++	++	−	−	−	−	−
Cucurbitaceae							
Cucumis sativa	+	+	−	−	−	−	−
Compositae							
Solidago canadensis	−	−	−	−	−	−	−
Cichorium intybus	−	−	−	−	−	−	−
Sonchus oleraceus	−	−	−	−	−	−	−
Taraxacum officinalis	−	−	−	−	−	−	−

Growth of embryo

At 30 hours after pollination a few zygotes have divided, and at 48 hours this is the modal condition. The endosperm often contains eight or sixteen nuclei at this time. Transverse division of the apical cell of the proembryo continues until a row of six cells is formed. The apical cell then divides longitudinally, giving rise to the first two cells of the embryo. The number of cells can be accurately counted up to 144 hours after pollination. A few divisions may occur in the suspensor during early differentiation of the embryo, leading sometimes to ten cells in this structure.

The counts on number of cells in the proembryo and embryo plus suspensor at six periods following self- and cross-fertilization are summarized in table 3. The data, like those on the endosperm to follow, were taken only on seeds showing no signs of disintegration. The mean values are the basis of the two growth curves in the lower part of figure 8.

Two facts are apparent from the data in table 3. In the first place, the number of cells in the embryo during the interval covered increases at approximately the same rate following both self- and cross-fertilization. The hybrid embryos may have a slight advantage, but the difference is not significant. Secondly, the growth curves depart only slightly from linearity; in other words, the number of cells increases additively.

TABLE 3

NUMBER OF CELLS IN THE EMBRYO FOLLOWING SELF- AND CROSS-FERTILIZATION

Time in hours after pollination	Type of mating	Number of cells in embryo																																													Total	Average
		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42		
30	Self	78	3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	81	1.04
	Cross	252	7	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	259	1.03
48	Self	33	79	4	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	116	1.75
	Cross	76	220	16	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	312	1.81
72	Self	...	16	20	26	8	4	3	..	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	77	3.65
	Cross	3	21	52	145	39	24	15	..	...	...	3	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	302	4.16
96	Self	...	1	2	8	5	7	44	..	3	..	5	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	75	6.63
	Cross	1	...	4	12	12	51	190	..	1	..	39	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	310	7.08
120	Self	...	...	...	...	...	...	17	..	2	..	16	2	2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	39	9.31
	Cross	...	...	...	...	...	...	73	..	5	..	116	4	14	..	3	18	...	...	1	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	234	10.32
144	Self	...	...	...	...	...	...	3	..	6	..	5	3	5	..	3	17	...	...	...	...	1	...	...	...	...	2	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	...	45	13.80
	Cross	...	...	...	...	...	...	6	..	...	..	45	4	20	..	10	103	...	4	11	3	13	..	2	...	...	28	...	2	...	4	...	...	...	5	...	...	...	2	...	...	...	3	...	265	17.28

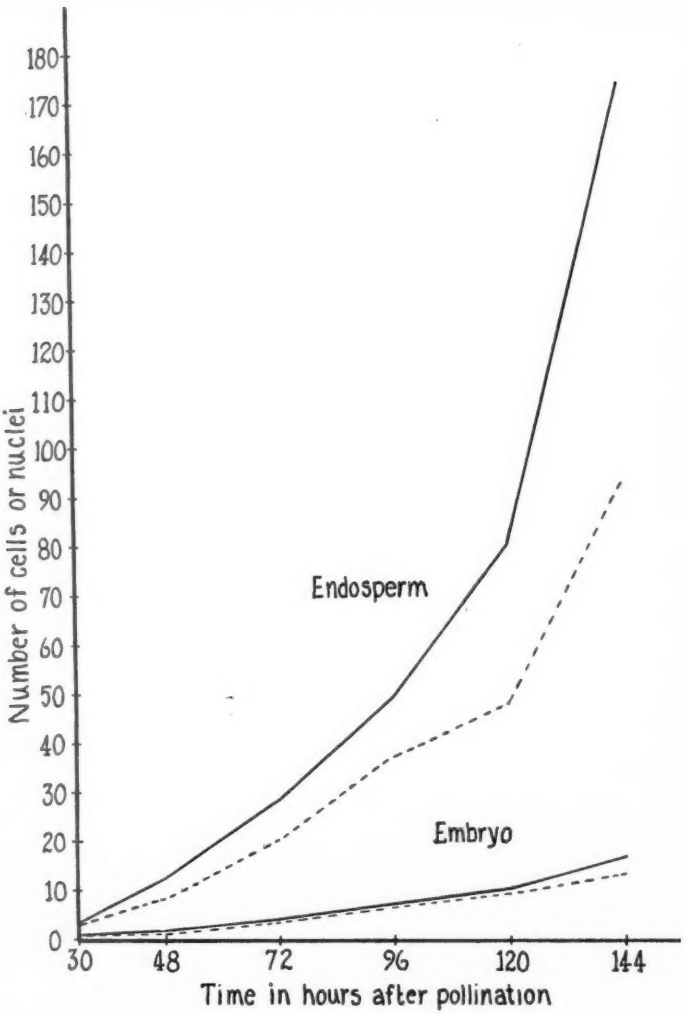


FIG. 8.—Increase in number of cells in embryo and in number of nuclei in endosperm following self- (broken line) and cross-fertilization (continuous line).

Growth of endosperm

The endosperm of alfalfa grows by free nuclear division during the period under observation. Only at 144 hours after pollination is the beginning of wall formation to be observed in a few seeds. Division is synchronous in the nuclei lying scattered throughout the cytoplasm. Starting at the chalazal end, a transient wave of mitosis progresses toward the micropylar end. Nuclear increase thus proceeds in a series of pulsations, as it were, each surge doubling the number. The significance of the rhythmic behavior is not known, but it demonstrates that the whole endosperm functions as a physiological unit at this stage, as might not be the case if the tissue were cellular. It may be noted also that division in unison is a mechanism admirably adapted to support inherent and dependent metabolic processes at a maximum potential. This characteristic of the endosperm may be important in relation to growth in the surrounding maternal tissue, as discussed later.

Growth in the endosperm was recorded in terms of the number of nuclei present. It might be objected that the data are not comparable with those from the embryo where cell formation occurs from the beginning. In a measure this criticism is well founded, and there is the further reservation that in neither case is the actual change in mass of tissue measured. Justification for considering that the bases used in comparing rates of development of embryo and endosperm are reasonably alike is seen in the fact that the latter shortly becomes cellular, the number of cells formed being equal to the number of nuclei present.

The data on growth of the endosperm following self- and cross-fertilization are shown in table 4. The mean numbers of nuclei present in the two series at 30, 48, 72, 96, 120, and 144 hours after pollination are plotted to form the two upper curves in figure 8. Except at 144 hours, the values were obtained by counting the nuclei present in each endosperm. At 144 hours the number is sometimes so large that it is not only difficult but also extremely laborious to determine. The difficulty is increased if cell-wall formation has started. The actual number of nuclei present was determined in the 144-hour material, however, in all cases where development of the embryo had not advanced beyond the 4-celled stage. This group comprised about one-third the seeds in the selfed series and about one-fifth those in the crossbred group. The number of nuclei in the remaining 144-hour seeds was estimated by averaging the counts from a part of the endosperms in each series distributed in accordance with the frequencies of the different embryo size classes. It is thought that the mean values thus obtained represent with sufficient accuracy for present purposes the stage of endosperm development in the more advanced seeds.

The fact has long been known that, following fertilization in angiosperms, the endosperm develops much more rapidly than the embryo. Few satisfactory meas-

urements of rate of endosperm development have been made, and we know of no other body of evidence as extensive as the present one. The data clearly confirm the established view that in rate of activity the endosperm far exceeds the embryo at this stage. A further conclusion may now be drawn. Whereas the number of cells in the young embryo increases approximately in arithmetical progression, the number of nuclei in the endosperm tends to increase geometrically. How long this relation is maintained in the growth of the alfalfa seed and whether it occurs in plants where the endosperm is cellular from the start is not known. As a

TABLE 4

NO. OF NUCLEI IN ENDOSPERM FOLLOWING SELF- AND CROSS-FERTILIZATION

TIME IN HOURS AFTER POLLINATION	TYPE OF MATING	NO. OF NUCLEI IN ENDOSPERM													TOTAL	AVERAGE
		1	2	4	8	16	32	64	96	128	192	256	384	512		
30	Self	11	30	37	3	....	....	....	....	....	....	....	....	....	81	3.0
	Cross	25	46	151	37	....	....	....	....	....	....	....	....	....	259	3.9
48	Self	8	4	16	67	21	....	....	....	....	....	....	....	....	116	8.2
	Cross	5	6	36	76	183	6	....	....	....	....	....	....	....	312	12.5
72	Self	....	....	5	8	37	26	1	....	....	....	....	....	....	77	20.4
	Cross	1	2	2	13	80	183	20	1	....	....	....	....	....	302	28.6
96	Self	....	....	....	1	15	38	21	....	....	....	....	....	....	75	37.4
	Cross	....	....	....	2	8	134	160	1	5	....	....	....	....	310	49.7
120	Self	....	....	....	....	4	18	13	3	1	....	....	....	....	39	48.4
	Cross	....	....	....	....	....	27	112	48	44	3	....	....	....	234	80.5
144	Self	....	....	....	....	....	4	14	11	12	2	....	....	....	43	93.0
	Cross	....	....	....	....	....	....	4	7	38	16	16	4	1	86	174.5

description of the present observations, however, it fits reasonably well. The implication of the fact is that of the two products of the fertilization process, embryo and endosperm, it is the endosperm which is the ascendant tissue in early development of the seed.

The relative development of the endosperm following self- and cross-fertilization may next be compared. As shown in figure 8, more nuclei occur in the hybrid endosperms (solid line) than in the inbred endosperms (broken line) at each of the intervals studied. Since the pistils collected each time necessarily were different, the data may be considered as comprising six independent sets of paired observations. If random sampling alone were operative in determining the sign of the difference in mean number of nuclei in the two series, one would expect the sign to be the same in all six cases only once in sixty-four trials. It is probable,

therefore, that the rate of growth of the endosperm following hybridization is significantly higher than after self-fertilization.

Increase in rate of growth of the young endosperm following entrance into the triple fusion of a male nucleus from another plant is a fact of importance in understanding double fertilization. Its full significance becomes clear when considered in relation to the histological changes associated with seed collapse just presented.

Number of endosperm nuclei associated with embryos at different stages of development

Fertilization after self-pollination in alfalfa may be somewhat deferred, owing to partial self-incompatibility. The delay is known to be rather brief. Nevertheless it may be long enough to raise doubt concerning the validity of comparing embryo and endosperm development after self- and cross-fertilization on a basis in which time of pollination is used as the zero point. Comparison of the growth relations independently of time as a coordinate is therefore desirable.

TABLE 5

No. of endosperm nuclei associated with embryos of given sizes after self- and cross-fertilization. Data based on 30–144 hour material

Stage of embryo development	Type of mating	No. of nuclei in endosperm										Total	Average
		1	2	4	8	16	32	64	96	128	192		
1-celled proembryo	Self	18	34	47	12	....	....	....	....	....	....	111	3.3
	Cross	30	52	183	64	2	1	....	....	....	....	332	4.3
2-celled proembryo	Self	....	....	10	63	26	....	....	....	....	....	99	9.7
	Cross	1	2	6	55	180	4	....	....	....	....	248	14.0
3-celled proembryo	Self	1	....	1	2	16	6	....	....	....	....	26	18.0
	Cross	....	....	....	8	43	21	....	....	....	....	72	19.8
4-celled proembryo	Self	....	....	....	1	18	15	....	....	....	....	34	22.8
	Cross	....	....	....	1	40	116	....	....	....	....	157	27.8
5-celled proembryo	Self	....	....	....	1	3	8	1	....	....	....	13	28.9
	Cross	....	....	....	....	2	41	8	....	....	....	51	36.4
6-celled proembryo	Self	....	....	....	....	5	5	1	....	....	....	11	27.6
	Cross	....	....	....	....	2	51	22	....	....	....	75	41.0
2-celled embryo+suspensor	Self	....	....	....	....	9	38	20	....	....	....	67	39.4
	Cross	....	....	....	....	2	106	159	14	3	....	284	54.0
3-celled embryo+suspensor	Self	....	....	....	....	....	2	9	....	....	....	11	58.2
	Cross	....	....	....	....	....	....	5	1	....	....	6	69.3
4-celled embryo+suspensor	Self	....	....	....	....	....	10	10	2	4	....	26	64.0
	Cross	....	....	....	....	....	11	114	28	46	4	203	83.7

The number of nuclei in the endosperm in relation to stage of development of the associated embryo is shown in table 5 and figure 9. The primary data are the same as those in tables 2 and 3, except that seeds in which the true embryo has advanced beyond the 4-celled stage are not included. As mentioned earlier, the number of nuclei in endosperms associated with embryos comprising five cells or more were estimated by averaging the values from a part of the seeds.

It will be seen from table 5 and figure 9 that at all nine stages of embryo development—zygote to 4-celled true embryo—the number of nuclei in the endosperm is

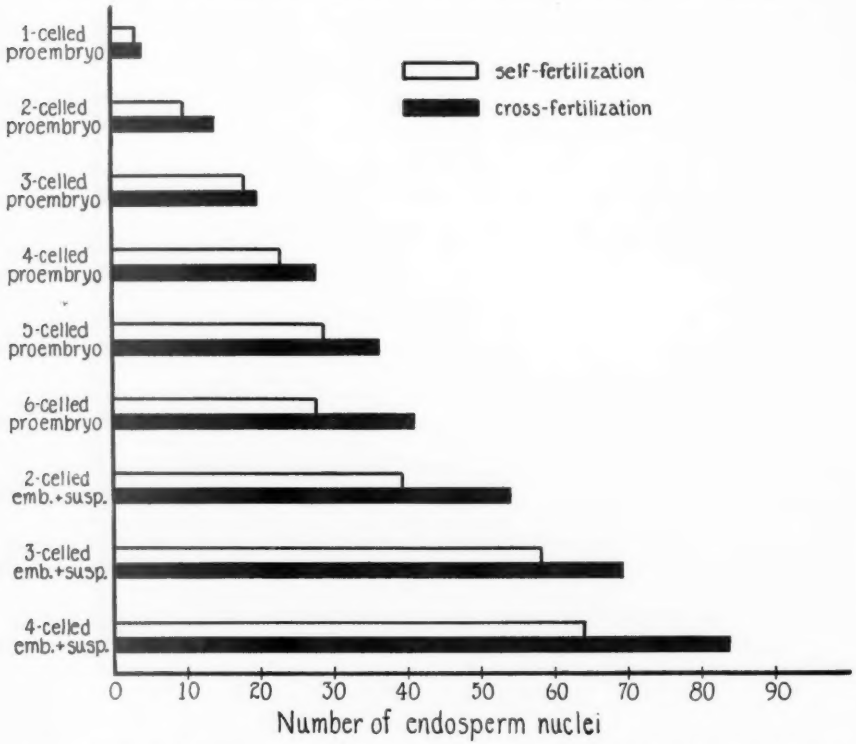


FIG. 9.—Number of endosperm nuclei associated with proembryos and embryos at various stages of development following self- and cross-fertilization.

larger after cross- than after self-fertilization. This evidence therefore confirms the conclusion already stated, that following hybridization the endosperm grows more rapidly than after selfing. Likewise it directly establishes the important corollary that, at a given early stage of development, a hybrid embryo is accompanied by a more advanced endosperm than is an inbred one.

Discussion

Double fertilization is of almost universal occurrence in angiosperms. According to SCHNARF (21), even in the few groups—such as the Helobiae and orchids—in which the endosperm may be regarded as suppressed, conjugation of the second male nucleus with the polar-fusion nucleus probably does not always fail. Permanency of the fully developed endosperm, however, varies greatly between species. The endosperm may be consumed by the embryo during development of the seed, as in most Leguminosae, including *Medicago sativa;* or it may persist as a constituent of the mature seed, as in the Gramineae, serving to nourish the seedling. There is general agreement that the endosperm is essentially a nutritive tissue, but its relation to the embryo in this respect during early post-fertilization development seems to have received little attention. The place of the fully developed endosperm in the economy of the seed is obvious, but the scanty attention given the tissue in embryological studies has allowed the more general and fundamental role in the young seed to remain in obscurity.

Four lines of evidence indicate that the endosperm of angiosperms exercises a primary and essential function in the early development of the seed. These may be summarized as follows:

1. Whatever its duration may be, endosperm tissue is regularly present at this stage. It is significant that in the rare exceptions to this rule, special devices may occur tending to serve the same end. In the Orchidaceae, for example, the suspensor of the embryo may serve as a haustorium, in certain cases growing out of the micropyle, branching, and imbedding itself in adjacent nutrient tissue. Noteworthy also is the fact that the embryo is poorly developed in this family.

2. Numerous investigators agree that immediately following fertilization the endosperm grows much faster than the embryo. The quantitative data obtained in the present study amply confirm this fact. As an auxiliary to the embryo, therefore, the endosperm makes prompt and extraordinarily rapid development.

3. The collapse of seeds in alfalfa, which commences within 48 hours after fertilization, is associated with reduced rate of endosperm development. Little difference is apparent in growth of the embryo.

4. The first signs of abnormal development in seeds which abort following interspecific hybridization appear in the endosperm. There is considerable evidence on this point, but mention is made here only of the work of BOYES and THOMPSON (1) on *Triticum* and *Secale*. These investigators found early endosperm development much more different than embryo development after reciprocal interspecific hybridization, and observe that "Whatever may be the primary cause of poor seeds and lack of success in crossing, it expresses itself through the endosperm."

In the other group of seed plants, the gymnosperms, double fertilization does

not occur. Its innovation in angiosperms becomes understandable when the conditions under which the endosperm functions are examined. The endosperm in gymnosperms is derived from the megaspore without alteration of the cell lineage and completes its development as a haploid tissue. The seed is nourished by the parent sporophyte in both groups, but the circumstances under which this is effected differ. A brief consideration of certain features of the reproductive process in gymnosperms and angiosperms will help to bring into clearer focus the *raison d'être* of double fertilization in the flowering plants. Attention centers on the local provisions for nourishing the embryo.

The extensive mass of undifferentiated tissue in the female gametophyte of the more primitive gymnosperms—the cycads, for example—constitutes a large nutritive reserve which is immediately available to the zygote. In other words, abundant food for the young sporophyte is at hand at the time of fertilization. In the higher gymnosperms there is progressive simplification of the female gametophyte. More important than this for nutrition of the embryo, however, is the general tendency—which runs through the whole group—to mature the egg earlier and earlier in the ontogeny of the gametophyte. COULTER and CHAMBERLAIN (7) summarize the matter in the following words: "In the most primitive condition of the gametophyte, the eggs do not appear until the endosperm is nearly full grown; and other gametophytes can be selected and arranged in a series showing the gradual slipping back of the eggs in the gametophyte, until in such a form as *Torreya* the archegonium initial is differentiated as soon as wall formation has taken place." This tendency reaches its climax in the angiosperms, in which the female gametophyte is reduced to a few cells, and the endosperm, as a definitive tissue, does not appear until after fertilization. One may logically infer that, in general, the endosperm of gymnosperms is sufficiently developed prior to fertilization to function immediately and directly in nourishment of the embryo. This mechanism might be expected to approach the limit of its effectiveness in the higher members of the group, owing to the great reduction of the gametophyte and the precocious differentiation of the egg. Although conclusive evidence is lacking, there are indications that this happens. The case of *Torreya taxifolia* may be cited as an example.

Torreya stands in an extreme position among gymnosperms in the tendency toward early development of the egg, although the endosperm consists of 400–800 cells at fertilization. In the mature seed of *T. taxifolia* is to be found a unique record of competition for the available nutrients between the developing endosperm and actively dividing cells just outside the megagametophyte. Ordinarily the endosperm in gymnosperms invades the surrounding tissue, the perisperm—more or less uniformly—consuming most of it. As described by COULTER and LAND (8), such invasion in *T. taxifolia* is extremely variable, leading to a highly irregular boundary between the two tissues in the mature seed. That the two

tissues are in competition is shown by the fact that growth in the one is in inverse relation to that in the other. The endosperm invades the tip of the nucellus, where the perisperm is not growing, obliterating all but a few peripheral layers of cells. Below the apical region, however, the perisperm is very active and resists invasion in many places. The greatest resistance is shown opposite the two vascular strands, where in the mature seed the endosperm is nearly cut in two by a deep constriction. The endosperm cells are radially elongated in regions of active invasion, and many of them are binucleate. Where the perisperm is less active, the endosperm cells are more nearly isodiametric and rarely binucleate.

There can be little doubt that in *Torreya*, proliferation of the perisperm restricts endosperm development. Carried somewhat further, the activity of the perisperm might entirely block endosperm development and thus deprive the embryo of nutrients necessary for growth. Sterility would then result. We have already shown that growth of the adjacent maternal tissues in angiosperms is a potential hazard to early endosperm and embryo development. The corresponding stages in *Torreya* seem not to have been studied, so that one cannot be sure that the phenomenon is homologous. The rather limited evidence from *Torreya* suggests that the endosperm as a strictly gametophytic structure nears its functional limits in the higher gymnosperms.

The relations discussed in the preceding paragraph may be looked at in a somewhat different way. COULTER and CHAMBERLAIN (7) emphasize that the endosperm is characteristically an aggressive tissue. Aggressiveness would appear to be a requisite for normal seed development, particularly where the young embryo is in competition with actively growing maternal tissue for a common nutrient supply. From this point of view, the margin which the endosperm must hold over the surrounding parts of the seed in order to insure nourishment of the embryo seems to approach the effective minimum in some of the higher gymnosperms. Further study of the early stages of seed development in the more advanced gymnosperms, such as *Gnetum* and *Ephedra*, would be of interest.

The angiosperms differ from the gymnosperms in three important factors affecting nourishment of the embryo: (1) The female gametophyte is reduced in size below that of the most advanced gymnosperms—in the typical case possessing only eight nuclei, one of which is the egg nucleus. (2) No endosperm is formed in angiosperms until after fertilization. (3) The angiosperm ovule contains little or no reserve food at the time of fertilization, in contrast with the considerable storage commonly occurring in the gymnosperms. As a result of these three circumstances the new sporophyte starts in an environment which might easily thwart its development. This follows from the fact that fertilization not only initiates development of the endosperm and embryo but also stimulates to active growth the previously quiescent cells of the surrounding maternal tissues. The lack of

reserves at fertilization and the non-appearance of storage products until the endosperm is well beyond the free nuclear stage mean that during this period the seed develops at a nutrient level which provides only for immediate tissue requirements. Translocated foods are used up as they are moved into the seed; there is no surplus. The much reduced gametophyte is small indeed relative to the total mass of the ovule. Originating at fertilization as simple uninucleate structures, embryo and endosperm must therefore compete for the limited nutrient supply with adjacent, well-established tissues of far greater extent. Starvation of the new sporophyte may thus easily occur if partition of nutrients within the seed gets out of balance. The success or failure of the young seed appears to hinge on the endosperm, as the nutritive agent of the embryo, establishing and maintaining a dominant position relative to the actively growing parts around it. Double fertilization, in the light of these considerations, emerges as a mechanism which enhances aggressiveness of the endosperm through the physiological advantages of the hybrid condition. It may also be thought of as a compensatory device tending to offset the disadvantage in reproduction associated with the extreme reduction of the female gametophyte in angiosperms.

The course of developmental events leading to seed collapse in alfalfa affords critical evidence in support of this interpretation. This type of seed failure, which we have called somatoplastic sterility (3), is attributed to inability of the endosperm to keep pace with growth in the surrounding tissues. Excessive meristematic activity in the latter results in starvation of the endosperm, and eventually abortion of the seed. The nutrient level immediately preceding and following syngamy is low, and probably the same foods are in concurrent demand by the protoplasts inside and outside the megagametophyte. The critical factor for survival appears to be the manner in which the nutrients are partitioned between the two regions. It is assumed that the available foods will be shared between the maternal tissues on the one hand and the endosperm and embryo on the other, in proportion to the velocity of growth in the respective tissues. If the rate of synthesis of nuclear material and cytoplasm in the endosperm falls below a certain minimum, which is frequently reached in alfalfa following self-fertilization, excessive growth is made in the inner integument or other adjacent maternal tissue, such as the nucellus, the endosperm is starved, and the seed collapses.

The occurrence of somatoplastic sterility reveals the delicate balance on which development of the seed in angiosperms hangs. Competition for nutrients may become acute between endosperm and maternal tissues. In order that the reproductive process be completed, a mechanism is demanded which will tip the scale in favor of the endosperm. Double fertilization is a method of conferring upon the endosperm the required advantage. It means that the endosperm of angiosperms

has two chances instead of one (as in the gymnosperms) of receiving the genetic equipment necessary to perform its function.

The differential survival of seeds in alfalfa following self- and cross-fertilization supports the hypothesis that the essence of double fertilization is a mechanism which secures to the endosperm the developmental advantages of hybridity.

The inequality of survival was determined by direct count of functioning and collapsing fertile ovules, the infertile ones being accurately separable. The increase in frequency of abortion after self-fertilization as compared with crossing within 144 hours after pollination was large, aggregating nearly fivefold. The difference characterized all seven plants tested. Since the two types of matings were made concurrently on each plant, the possibility of a factor other than the constitution of the male nuclei being responsible for the difference in survival is ruled out. One may think of the experiment as involving introduction into a common series of megagametophytes of the two classes of sperm as reagents. A reaction favorable to survival of the seed is evoked by one class of sperm (from an unrelated plant) in 93 per cent of the cases and by the second class (from the same plant) in only 66 per cent.

The reaction called forth by the two classes of sperm may next be considered. The rate of growth of embryo and endosperm derived respectively from the two products of fertilization was measured up to 6 days after pollination. Normal appearing proembryos and embryos are formed following both types of matings; if there is any difference in average rate of development at this stage it is too small to be established from the present data. The endosperm develops with great rapidity relative to the embryo, and it is in this tissue that a significant effect of cross-breeding is manifested. From the time of the first observations onward, the hybrid endosperm has a higher average number of nuclei than the inbred endosperm. Furthermore, the mean number of endosperm nuclei associated with the embryo from the zygote to the 4-celled true-embryo stage is regularly higher after hybridization. There is little reason to doubt that the added impetus to growth of the endosperm resulting from cross-fertilization is the cause of the higher survival. It may be supposed that a certain minimum rate of endosperm activity is essential to continued development of the seed; otherwise failure occurs through hyperplasia of the adjacent maternal tissues. Following self-fertilization, this rate of endosperm growth is attained in significantly fewer instances than following crossing.

The generally advantageous effects of cross-breeding on development of the organism are too well known to require more than brief mention. Heterosis is expressed in manifold ways, and apparently at any stage in ontogeny. It is well known in the mature endosperm through the work which has been done on maize. COLLINS and KEMPTON (4) found that hybridization with unrelated strains

increased the size of the ripe seed from 3 to 21 per cent in different *Zea* crosses. The existence of heterosis in the growing endosperm of alfalfa is demonstrated directly in the present investigation. The data are unique, so far as we are aware, in showing that hybrid vigor may be expressed as increased rate of nuclear division starting with a fertilization product.

LANDES (16) has shown that reduced seed formation in rye following self- as compared with cross-pollination is due, not only to a lower frequency of fertilization, but also to the collapse of fertile ovules. In these respects rye closely parallels alfalfa, as described earlier (3). LANDES found that abnormal development of the endosperm is common after self-fertilization. It seems probable, therefore, that seed collapse in rye, as in alfalfa, is associated with impaired capacity of the endosperm for growth. Heterosis in the endosperm following cross-breeding may be assumed to favor continued development of the rye seed, whereas the frequently occurring collapse of fertile ovules after selfing is best interpreted as an inbreeding depression effect.

The decreased rate of endosperm development in the self-fertilized alfalfa series is probably accounted for by the bringing together in homozygous condition of certain recessive genes with deleterious effects. KIRK (15) has shown that the vigor of the adult alfalfa plant is sharply reduced by self-fertilization, and the writers' observations on many families are in agreement. Defective individuals are also common in inbred families, such as seedlings with reduced chlorophyll production. It might be expected, therefore, that recessive genes adversely affecting the endosperm are also present. Since a single generation of self-fertilization reduces heterozygosity 50 per cent, the frequency of zygote and primary endosperm nuclei carrying such genes in homozygous condition is greatly increased in the self-pollinated series.

Aside from the fact that there are twice as many genes present, the nuclear condition to which inbreeding a diploid plant leads is like that prevailing in a haploid: the inhibitions resulting from dominance disappear, and the deleterious recessive genes exert their full physiological effects. In view of the sharp increase in failure of seeds in alfalfa accompanying a 50 per cent reduction in heterozygosis, it is apparent that fertility would be drastically impaired if the endosperm were haploid, as in gymnosperms.

The possibility is not excluded that partial self-incompatibility, per se, adversely affects seed development after self-fertilization. EAST (12) tested fifteen genes controlling self-sterility in *Nicotiana*, however, and found that none of them in homozygous condition weakened plant development. It is unlikely that fertile ovule survival is influenced by self-incompatibility as such.

It is necessary only to mention in passing the question whether the depressing effect of inbreeding on endosperm development in alfalfa is conditioned by a few genes

with conspicuous effects, many genes of minor influence, or both. MANGELSDORF (17) has identified a number of major genes in *Zea* causing defective endosperm. Those which give a viable plant in homozygous condition have markedly unfavorable action likewise on general development. In other words, these genes affect some basic physiological processes. Corn breeders recognize variation in endosperm development in highly selected inbred lines, which is probably attributable to the combined action of many genes with slight effects. It is likely that numerous genes, varying in magnitude of effect, are concerned with endosperm development in alfalfa.

Cross-fertilization, although not universal, is the prevailing mode of reproduction in flowering plants. EAST (11) and others have pointed out the broad evolutionary advantages it confers upon organisms as a concomitant of sex. The benefits of cross-breeding in relation to development have been demonstrated in many species, JONES' (14) work on corn being a classic example. Hybrid vigor in the endosperm, of course, cannot be a factor in seed formation in regularly self-pollinating species. If our interpretation of the role of the endosperm is correct, however, genes adversely affecting endosperm growth will tend to be automatically eliminated in autogamous forms.

In view of its generally invigorating effects, it is not surprising that hybridization should prove to be an important factor in completing certain processes ancillary to reproduction in flowering plants. Double fertilization, to be sure, is a novel means of extending the advantages of hybridity, but the end gained is clear enough. By this device the endosperm, playing a brief though vital role in development under unique circumstances, acquires a significant share in the benefits of sexuality.

Summary

1. Following self-pollination in alfalfa, 34.4 per cent of the fertile ovules collapsed during the first 144 hours as compared with only 7.1 per cent after cross-pollination.

2. Fertile ovule collapse is associated with excessive growth of the inner integument. The first signs of abnormal development occur in the cells adjacent to the megagametophyte on the funicular side of the seed in the region of the vascular bundle. Breakdown of the endosperm follows, and the seed eventually aborts.

3. This type of seed failure is attributed to inability of the endosperm to keep pace with growth in the surrounding tissues of the seed.

4. The number of cells in the embryo up to 6 days after pollination increases in arithmetical progression. There is little difference in rate of growth following self- and cross-fertilization.

5. The number of nuclei in the endosperm increases exponentially during this period.

6. The rate of growth of the endosperm following hybridization is significantly higher than after self-fertilization.

7. The number of endosperm nuclei associated with an embryo at a given stage of development is regularly higher following cross- than following self-fertilization.

8. A survey of thirty-six species, in addition to alfalfa, shows that the angiosperm ovule is characteristically low in food reserves at fertilization. Early development of the seed is therefore dependent upon translocated foods. The endosperm is considered to play an essential role in the partition of these nutrients.

9. The evidence on differential survival of seeds following self- and cross-fertilization, growth of endosperm and embryo, histological changes associated with collapse, and comparative lack of ovule food reserves are considered in relation to the role of double fertilization in seed formation.

10. Conjugation of a male nucleus with the polar-fusion nucleus to form the primary endosperm nucleus is interpreted as a mechanism by which the physiological advantages of hybridity become available to the endosperm. Continued development of the young seed demands a rate of endosperm development which is attained more frequently following cross-fertilization by virtue of heterosis than following self-fertilization. Confirmation of this view is seen in the behavior of alfalfa seeds following the two types of matings.

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NEW LEPIDOSTROBI FROM CENTRAL UNITED STATES

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 514

G. B. MATHEWS

(WITH SEVEN FIGURES)

Introduction

Knowledge of the structure of *Lepidostrobus* is based mainly on material discovered in Europe. The incrustations, of which many were described from the United States, furnish information concerning the external appearance of the organ or plant. Only three or four American species are recorded which were petrified and possible for anatomical study.

COULTER and LAND first described an American *Lepidostrobus* in 1911 (**4, 5**). The cones were found in Pottsville strata, near Indianola, Warren County, Iowa. In 1921 these investigators reported other cones from the same locality, but they could not expand the earlier description since the structure of the axis was not preserved in all the material. These specimens are now mainly in the collection of the Department of Botany at the University of Chicago, and are numbered: G21, P1–P11, and 502 (BF1, 21). JONGMANS (**8, 9**) named the cone described by COULTER and LAND *L. coulteri*. This *Lepidostrobus* is considered homosporous. In 1914 another species, *L. fischeri*, was described in detail by SCOTT and JEFFREY (**13**) from the Mississippian. It was later named *L. kentuckiensis* (**14**), since it was found in Kentucky, but the name which had been given it was preoccupied. In 1935 GRAHAM (**6**) described two kinds of small lepidostroboid cones from the Upper Connemaugh. These were found in coal balls from the Calhoun coal mine, Richland County, Illinois. Although these species are distinct from others hitherto described, the poor preservation of details led GRAHAM to leave the species unnamed.

It seems apparent that the cones upon which this study was made are of the same species as those described by COULTER and LAND (**4, 5**). The macroscopic features correspond well; the microscopic structures are the same, although COULTER and LAND could not give a description of the axis because it was not preserved. The cone with the best preservation is a cotype, G21 M1–M5. Another cone of which there are two sections in the collection is numbered G21 N1 and N2, and of the type specimen as described by COULTER and LAND (**4**) several slides have been made, G21 P1–P11. The variations in size of leaf traces as found in G21 M and G21 N and the differences in the structure of the lamina seem to be within the range of the same species.

Lepidostrobus coulteri Jongmans

Diagnosis

Cone large, 5–6 cm. in diameter; length up to 22 cm.
Axis 8–9 mm. in diameter.
Stele with central undifferentiated tissue; xylem 400–500 μ in thickness.
Leaf traces collateral; in outer portion of middle cortex 400–600 μ in diameter.
Ligule present; heel about 3 mm. long.
Parichnos present; bifurcates and branches, entering lamina.
Cortical layers about 3000–3400 μ thick.
Sporangium basal; palisade cells enlarged at corners.
Spores usually in tetrads of 52 μ, single spores 27 μ.
Lamina 12 mm. at base, $\pm$ ridged, 24 mm. long.
Geological horizon: Pottsville.

The following description is based on five sections in the collection of the Department of Botany, University of Chicago, G 21, M1–M5. This previously undescribed specimen was found years ago with other cones in a coal pocket near Indianola, Iowa, the same locality from which the specimen described by Coulter and Land came. The geological horizon is Pottsville.

This specimen represents only a portion of the entire cone. The five thin sections show the anatomical features fairly well. A radial section through the upper portion shows the stem axis with stele and cortex and the arrangement of the sporophylls (fig. 1*A*). Only a few remnants of the laminae can be seen. Of the other sections, one is median longitudinal (fig. 1*C*), showing arrangement and position of structures in and outside the stele. From it the spiral arrangement of the sporophylls, shape of the heel and lamina, and many microscopic structures can be seen. The other longitudinal sections are more or less parallel to the median one. One section is slightly oblique, passing from the outer cortex at the lower part through the middle cortex and almost into the inner cortex at the upper part. A section parallel to this last shows sporophylls, pedicels, and sporangia in oblique view at the upper part and the leaf traces in the outer cortex at the lower part. Another tangential section is through sporophylls which are at right and oblique angles.

The following data about the size of this cone are based on these sections, since no record was available as to the actual size of the entire cone. The longitudinal sections are at most 51 mm. long; with the upper end, which had been cut off, the entire fragment was about 7 cm. long. One side of the cone is not preserved, thus the diameter can be estimated only from the known radius, which is 4 cm. The diameter of the axis tapers from 9 to 8 mm., which suggests a rather large cone, probably not less than 20 cm. in length. The shape is cylindrical. Calcium car-

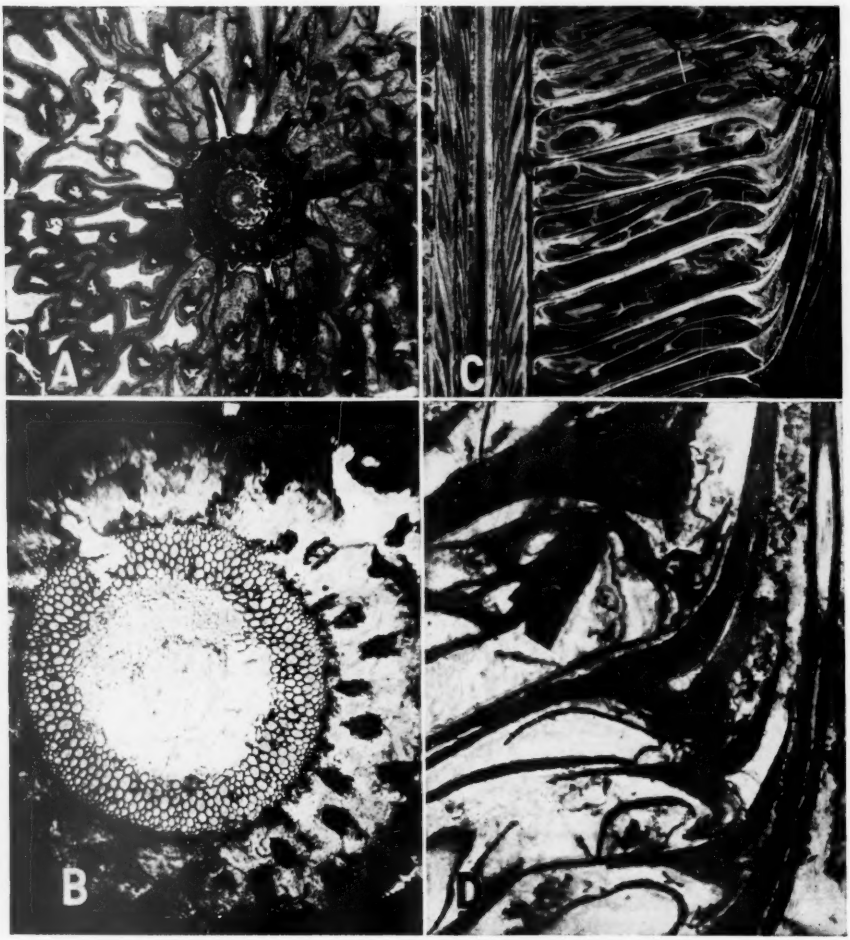


FIG. 1.—*A:* cross section of *Lepidostrobus coulteri* showing axis and sporophylls; G21 M5. *B:* part of *A*. Central portion of axis showing stele with protoxylem and metaxylem, central tissue in upper part, and empty space between stele and inner cortex. *C:* longitudinal section showing axis with tissues and arrangement and structure of sporophylls; G21 M1. *D:* part of *C* enlarged to show heel, parichnos, and leaf trace in lamina.

bonate was the main fossilizing agent. Other minerals, such as pyrites, were not noticed.

The lower and upper portions of the cone can be distinguished on the basis of several criteria: direction of the leaf traces in the axis, arrangement of the sporangia, relation between the pedicel and sporangium, location of the heel on the abaxial side of the pedicel, upward direction of the laminae, and other microscopic features. The spores were shed before fossilization, but here and there some are found in many sporangia. These spores are all of the same kind and apparently *in situ*.

Cone axis

The cone axis is cylindrical and has a diameter of 9 mm. at the lower end. It tapers a little to 8 mm. at the upper end in the distance of 51 mm. The radial and longitudinal sections show poor preservation of the central tissue, good preservation of metaxylem and protoxylem and of the inner and outer cortex, and fairly good preservation of the leaf traces in the stem axis. The core of the stele is not preserved except for some elements which are in contact with the metaxylem. These latter elements are of the same size as the mature metaxylem elements, 50–150 μ in diameter. All these cells have distorted cell walls, and of those most centrally located only the corners of the cells are preserved. The differences between these centrally located cells or "primitive fibers" and the metaxylem are that the former have little secondary wall thickenings and the end walls are at right angles to the longitudinal axis. While there are these slight differences, many similarities lead to the conclusion that these cells are to be regarded as undifferentiated metaxylem cells.

Adjacent to these central cells are the larger elements of the metaxylem. They have a diameter of 30–120 μ, while the thickness of the whole belt is 400–500 μ. The long section shows that there were eleven scalariform thickenings per 100 μ of length in the central part and that the number of thickenings increases peripherally to fourteen and twenty-two per 100 μ. The metaxylem elements gradually merge into the smaller elements of the protoxylem strand. The spiral thickenings of these latter are closer together, about thirty-six per 100 μ. The gradual transition is shown also by the decreasing diameter of the metaxylem elements and transitional forms of the thickenings. No longitudinal striae between the thickenings were noticed.

The cylinder of tissue just peripheral to the xylem is not well preserved. It is about 300–400 μ in thickness. A few remnants are present here and there, of small diameter (12–30 μ) and parenchymatous. The space occupied by this layer is the one in which the endodermis and the pericycle might be differentiated, but these structures could not be clearly discerned. The leaf traces are preserved in this belt.

The cortex, which is differentiated into an inner, middle, and outer portion, thus resembles many forms already described. The inner cortex is bounded centrally by the inner space just mentioned. The crenulated outlines of the inner cortex are associated with the leaf traces, which extend through this tissue. A tangential gap occurs (fig. 1*B*), the total width therefore varying from 600 to 1000 μ. The polygonal cells are of various diameter, from 30 to 75 μ, with a length of 110 μ. From the longitudinal sections it is apparent that the cells of the inner cortex are not parallel to the strands in the stele in their longitudinal axes. The cells (or better, the cell walls of the inner cortex) gradually merge into the empty space peripheral to the inner cortex. The middle cortex, which was present where the space now occurs, is rather thick, almost 2 mm. No trace of any structure of the tissue formerly occupying this space could be detected. As in other forms, perhaps this trabecular tissue was too delicate to withstand the decaying influences before fossilization took place. From the rootlets and other structures of foreign origin present in the specimen, it is clear that fossilization took place a considerable time after the cone was detached from the tree. The parichnos, however, which extends from the middle cortex, may reveal something about the cellular peculiarities of this part of the cortex.

The leaf traces are well preserved as they extend through the middle cortex. The outermost portion of the stem axis is the outer cortex, a rather dark sclerenchymatous tissue 400–800 μ in thickness. Two kinds of cells can be distinguished. The inner ones are tangentially elongated and radially flattened, 25–30 μ × 12–15 μ. Several layers farther out the cells are isodiametric and spherical. In general the outer portion of this tissue is rather dark, and few details can be discerned. The lumen of these cells is 12–10 μ. This layer abuts the bases of the sporophylls, the pedicel.

The sporophyll is composed of the pedicel and the sporangium (fig. 1*C*). The maximal length for the pedicel up to the heel is 22 mm. The pedicel and with it the sporangium has a slightly upward direction, but the angle is not the same at both sides of the axis, owing to unilateral pressure. The pedicel is of triangular shape where it leaves the axis. For a short distance it is not connected with the sporangium, since no attachment of the sporangial tissue can be noticed and the epidermal layer is continuous around the pedicel. As the pedicel continues on the way out, it flattens and enlarges on both sides. Almost at the point where the sporangium ends, a strong downwardly projecting heel 2.5 mm. long is developed. The pedicel continues in a straight upward direction as lamina. The shape and size of the lamina are perceptible from several sections. The length is 24 mm., with a maximal width at the base of its origin of 12 mm. From the fact that each lamina shows a markedly enlarged portion on its section it is concluded that it had an abaxial ridge in which the vascular bundle was located. The blade of the lamina

is in some places 150 μ thick, while the vein has a thickness of 400 μ. The thickest lamina was about 800 μ at a distance 8 mm. from the heel base. The pedicel thus enlarged from 1.3 mm. at the very beginning and 3 mm. at the base of the heel to a lamina 12 mm. in width and a total length of 46 mm.

Pedicel

The heavy sclerized cortex is continuous with the pedicel. The sclerotic tissue surrounds the vascular bundle and the parichnos on all sides except in the region of attachment of the sporangium. In this latter area the adaxial epidermal and hypodermal layer is replaced by another tissue for almost the entire length of the sporangium. The tangential width of this connective tissue varies with the size of the pedicel at that special point. Usually it is in a relation of 2:5. From this tissue arise the sporangial wall, the tapetal tissue, and the subarchesporial pad. The heel has a peculiar structure. On the abaxial side no strong epidermal layer is present. The subepidermal layers have been destroyed up to 400 μ into the tissue, and the empty space shows that this tissue was probably of delicate nature, although the epidermal layers show a wavelike contour over the heel. They assume the usual strong habit of dark epidermal and subepidermal tissue after 3–5 mm. The heel is filled with parenchymatous cells. The cells below the parichnos are of various size and extend at a right angle to the leaf trace but are arranged parallel to one another. The tissue which separates the leaf trace and the parichnos is more regularly arranged in vertical and horizontal rows several layers in thickness. The trapezoidal cells are 50–80 μ long with a median diameter of 25 μ. On the adaxial side of the heel the hypodermal layer is built up of rather long sclerenchymatous cells, 300–400 μ long and 20 μ broad. In the lamina the cells are isodiametric, especially those near the parichnos, and resemble cells of mesophyllous character. Small intercellular spaces are interposed among the cells adjacent to the parichnos.

Sporangium

The sporangium is attached to the adaxial side of the pedicel and is 22 mm. long, 4 mm. high, and 5 mm. broad. The sporangia are mostly empty, although in many of them a few spores remained in the distal and proximal parts. By the flap at those parts pouches are formed which probably inhibited the spores from escaping. These spores are found in almost all parts of the cone, in the lower as well as in the upper section. The sporangia were ripe when fossilization took place, and the sporangia open, but some structures permit certain conclusions about the shape of the mature sporangium. On each lateral sporangial wall are two points where the cell walls are larger and functioned apparently for the stiffening of the wall. These enlarged palisade cells appear distinctly at almost the same loci in the cross-sectioned walls. They indicate the corners of the sporangial bag. The first thickening

is near the base at a distance which corresponds to the length of the wing of the pedicel. The next is about 1 mm. or more in front. After this follows the last portion, which is always a little longer than half the width of the relative pedicel at that point. Thus the mature sporangium would be of the usual shape, with a thickening on the four corners. The palisade cells are in general 75 μ high. At the corners they reach 125 μ but decrease toward the point of dehiscence to 25 μ, while the diameter remains 16 μ. The inside of the sporangium is lined with the tapetal layer, which is gone except in some instances at the distal or proximal pouches. These thin-walled elongated cells line the sporangium wall, sometimes eleven cells deep, to a total thickness of 140 μ. The subarchesporial pad is composed of extremely delicate cells. No definite arrangement of sterile tissue plates was noticed.

Spores

All except a few spores were shed. They are mainly single but in some instances are arranged in tetrads. Their surface is simple. The diameter of a tetrad is 52 μ, while the largest diameter of a single spore is 27 μ. The fact that they are of the same size and shape at the lower as at the upper part of the cone fragment leads to the conclusion that the cone was a staminate one or the plant homosporous.

Leaf traces

The arrangement of the leaf traces in the axis and sporophyll is visible in the long and cross sections (fig. 1*A*, *B*, *C*). The bundles take their origin from the points of the peripheral protoxylem. At the point of departure from the vascular cylinder there are about nine elements present, not surrounded by any preserved tissue. As the bundles go farther away from the stele, a surrounding sheath develops. These cells have a brownish appearance, while the xylem cells have a whitish crystalline content. The cells which compose the sheath are longitudinally elongated, with walls at right angles. The shape of the leaf trace in the inner space is radially elongated in cross section but becomes cylindrical as it gains distance from the stele. The number of xylem elements increases to about twenty-three but is not constant. The smaller protoxylem elements are usually in a mesarch position and number about seven.

With the approach of the leaf trace to the inner cortex, the appearance of a phloem portion in the trace becomes apparent. Where the leaf trace nears the inner cortex the tissue of the latter recedes, bulging away from the trace. At the same time the tissue which surrounds the trace sends out two lateral peripheral projections. These unite with the inner cortex, leaving between them an empty space which probably had been occupied by the phloem. A phloem strand in connection with the leaf trace cannot be seen in the inner space, probably because this belt was filled with parenchyma, which was not preserved. The vascular bundle

shows a different structure in its passage through the inner cortex. An empty space accompanies the leaf trace on its abaxial side. A sheath surrounds the bundle as a whole and the xylem strand which is separated from the phloem by the conjunctive tissue.

The delicate phloem tissue is not preserved at any place in this fragment—except for a small portion which probably had been broken off before fossilization began and thus had a chance to undergo mineralization by quick infiltration before decay started. The space occupied by the phloem in the inner cortex is cylindrical. Its tangential diameter decreases peripherally, while the radial one increases. Conjunctive tissue separates the xylem from the phloem. A different sheath surrounds the whole bundle as it passes through the middle cortex. This sheath is of the same structure as the tissue of the inner cortex. On the whole, except for size, the leaf trace in the middle cortex has the same arrangement and structure as in the inner cortex. In the middle cortex only the leaf trace is preserved, without a trace of the surrounding tissue. As in entering the inner cortex, the tissue of the outer cortex bulges back where the leaf trace approaches, but no projections of the sheath are formed. The clear phloem space in the bundle becomes flattened radially until it is crescent-shaped. A few remnants of cell walls on the periphery of the empty space indicate cells of small caliber. Another empty space appears in the outer cortex. It lies abaxial to the bundle as a continuation of the tissue of the middle cortex. In the same way that the phloem became apparent only in the next belt, the inner cortex, and the cortical bundle sheath in the middle cortex, so the parichnos shows up in the outer cortex, which is the following tissue. The soft delicate tissue is not preserved. The tangential width of the parichnos is the same as that of the whole bundle. The radial diameter is almost the same as that of the phloem. The total length of a leaf trace in the stem axis from the point where it leaves the protoxylem ring until it enters the pedicel varies from 1.5 to 2.5 cm.

These data are not taken from a single bundle, since none is cut longitudinally its whole length; they are based upon measurements of various leaf traces in different tissues of the cylinder. The course of the leaf trace is shown in the longitudinal axis. All leaf traces are cut in the same plane. On the upper end the traces are just leaving the inner cortex. A rather large portion is cut, about 3 mm. It runs parallel to the plane of cutting, while in the outer cortex the cut leaf trace measures almost 1 mm. in length, although enlarged during the journey through the cortical layers. The explanation is that the course of the leaf trace is rather steep in the beginning but becomes more horizontal in the outer half of the outer cortex. In the pedicel the position of the leaf trace is on the adaxial side close to the sporangium. Surrounding the leaf trace in the heel are the barred cells of the transfusion tissue on the adaxial side. These cells run not only parallel to the leaf trace but also at right angles to it. They accompany the leaf trace into the lamina and

are located laterally to it. There is no space left on the ventral or dorsal side because the trace fills out the space completely. An abaxial keel is formed which is rather prominent in comparison with the thin blade of the lateral wings of the lamina. At a distance of 22 mm. from the heel base, a leaf trace was noticed about 300 μ thick. There three xylem strands could be seen surrounded by a mass of thin-walled elongated cells with oblique walls.

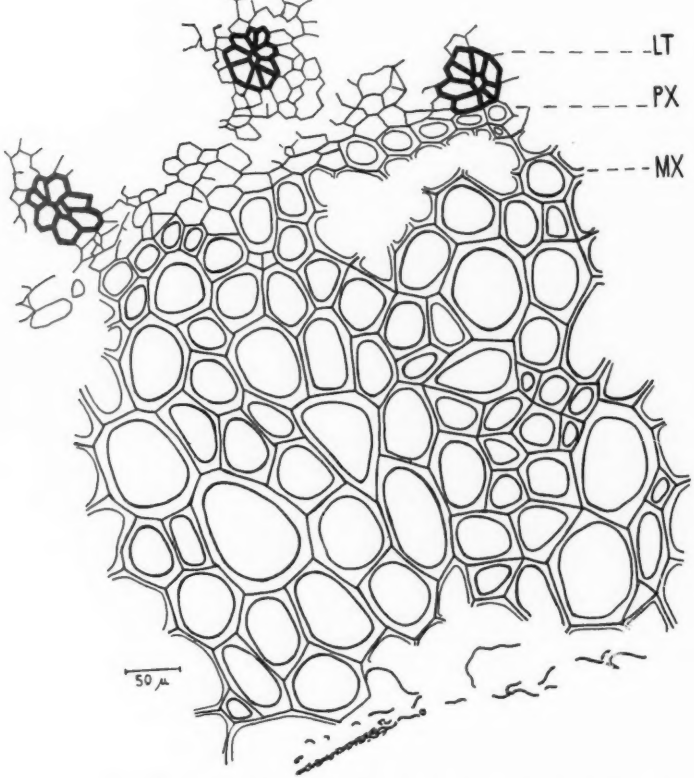


FIG. 2.—Axis of *Lepidostrobus coulteri* showing decayed central tissue, metaxylem, and protoxylem (*mx*, *px*), and some leaf traces (*lt*); G21 N1.

PARICHNOS

The parichnos extends from the middle cortex and is first noticed in the outer cortex on the phloem side of the leaf trace. It connects the lacunar tissue of the middle cortex with the intercellular spaces of the lamina. At about two-thirds the

length of the pedicel the parichnos splits off into three branches. The median one is at first bigger but soon decreases and after a short distance fades out, while the two lateral ones continue. In the heel the parichnos has a radial or tangential diameter of 300–500 μ but decreases rather rapidly in width (fig. 1*D*). In the lamina it is surrounded by cells with intercellular spaces, two layers on the abaxial and one on the adaxial side. The space taken by the parichnos corresponds almost to the width of one cell layer of the surrounding tissue—30 μ.

Two other mounted sections were found in the collection, G21 N1 and N2. One is a complete radial section; the other shows about a quarter of a radial section with the entire axis. These sections are (*fide* Dr. A. C. Noé) from the same locality as the first cone described. Both sections are no doubt from the same specimen. The external appearance suggests the same composition as that of G21 P, but in this specimen pyrite crystals fill the cells here and there.

The diameter of the cone is 6 cm., laminae included. The axis measures 8 mm.; the distance between the axis and the lamina is 2 cm. The central tissue is not preserved except for a few remnants. These are compressed and show as a rather sharp line separating the empty central space 1.3 mm. from the xylem, which is 500–600 μ in thickness (fig. 2). The next zone is not preserved except for a few cells bordering on the protoxylem, and the leaf traces.

An approximate estimate can be given for the thickness of the space between xylem and inner cortex. From the preceding description it is evident that lateral projections extend peripherally from the leaf traces as they approach the inner cortex. The projections are preserved, the tissue of inner and middle cortex being destroyed. The space between xylem and inner cortex is about 600 μ thick. The following space, occupied formerly by inner and middle cortex, measures 2100–2400 μ. The leaf traces near the outer cortex measure 400 × 500 μ, with a phloem space of 175 × 75 μ. They are smaller than those of the previous cone, with 600 × 600 μ and a phloem space of 200 × 150 μ. The laminae are known only in cross section. The width of the lamina in the first peripheral row is 12 mm. Farther out in the third row some are 1800 × 500 μ and 700 × 300 μ. The middle vein is not sharp. The few spores measure 25 μ. They are shrunken and attached to the sporangial wall.

Lepidostrobus noei sp. nov. Mathews

Diagnosis

Lepidostrobus noei Mathews, centimeter 15 longus, 7 crassus, bracteis millimeter 8–20 longis, laminis medio-costatis millimeter 14 longis, 8 latis; sporis dimorphis magnitudine comparatis ratione 1:5, microsporis minutis quaternatim conjunctis millimeter 0.050; macrosporis millimeter 0.275–0.330 innumerabilibus sporangia inferioris tenentibus.

Cone large, heterosporous, 7 cm. in diameter and more than 15 cm. long (actual length of fragment 11 cm.).
Axis without central tissue, 7 mm.
Stele with central tissue of undifferentiated xylem; xylem layer 300–400 μ thick.
Phloem surrounding the xylem.
Pericyclic fibers present.
Leaf trace collateral; xylem elements more than thirty; diameter of leaf trace (with slight variation) 100 μ in axis.
Parichnos not extending into peripheral part of outer cortex.
Sporangia basal, five-sided polygon, sterile plates short, unbranched.
No heel present.
Megaspores numerous in sporangia at base of cones, smooth, with triradiate ridge, 275–330 μ.
Microspores in sporangia at top of cone, tetrads 76 μ, single 50 μ.
Laminae 8 mm. broad, with sharp vein; 14 mm. long.
Geological horizon: Upper Devonian (?).
The new cone is named after the late Dr. A. C. Noé.

This cone was found near Gartersville, Gorrand County, Kentucky. It was forwarded to Dr. A. C. McFarlan of the Department of Geology at the University of Kentucky, Lexington. Dr. A. C. Noé, University of Chicago, to whom the cone was sent for classification, gave it to the writer for a superficial study. When it was found that the cone was heterosporous, all efforts were made by Dr. Noé to get permission from the respective authorities in Lexington to section the cone and make a detailed study. The sections and slides are now in the Department of Botany at the University of Chicago, nos. G22 M1–M33. A big fragment showing a long section and partial radial section with the external structure intact is now in the Department of Geology, University of Kentucky, no. M3121. As to the geological horizon, Dr. McFarlan wrote that the cone was found "on the edge of the Knobs, which would make possible its occurrence in either Ohio or New Providence shales. It is unfortunate that the precise horizon is not known." The Ohio shale would place the cone in the Upper Devonian; the New Providence shale, in the Mississippian. The New Providence shales contain only marine fossils, as is known until now. Before any sections were made, some excellent molds were made in plaster of paris by the courtesy of the Daprato Statuary Company, Chicago, Illinois.

General description of cone

The cone is a petrified specimen and is almost free from adherent foreign material, so that the external structure can be seen. The color on the outside is gray and inside, brownish. At the lower part a greenish taint was caused by the pres-

ence of algae and protonemata of mosses. The fructification was exposed to climatic influences for considerable time. On the lower part a few sporophylls were broken off. On one side the laminae are not present for almost the entire length of the cone. Fossilization of the cone is no doubt excellent; the mineral, however, is mainly calcium carbonate and is decayed to a large extent. Peels do not therefore reveal the details. The observations and pictures were made mostly with reflected light. Some of the megaspores were replaced by siliceous compounds, others by pyrites. The connective tissue between pedicel and sporangium is not preserved but is replaced by transparent calcites.

The length of the fragment is 11 cm. (fig. 3*B*). The maximal width is 7 cm., about 8 cm. from the lower end. The cone is wider at the middle, tapering toward the top and bottom. By pressure of unknown forces the cone—and with it the axis—was deformed from a cylindrical to an elliptical shape, so that the diameter varies. The small diameter is 5.5 cm.; the large one is 7 cm. Compression occurred in such manner that on one side the laminae were appressed and the cone broke longitudinally. The halves were disarranged, so that on the same radial section the cone axis on one side measures 2.5 cm. in thickness (except the central tissue) and on the other side 3.1 cm. The weaker central tissue is mostly crushed. All laminae have been broken just at the point where one would look for the ligule. Where the laminae had been broken off before fossilization, decaying processes have exposed spores and the structure of the pedicels. The spores could be seen with the naked eye and filled the sporangia in great masses; surely megaspores. A small piece of a broken sporangium from the upper end contained spores much smaller than the megaspores in the sporangia below. In the same uppermost whorl were also some megasporangia; thus this whorl consisted of mega- and microsporophylls.

An upper part, 1.8 cm. in thickness, was cut off radially to study the arrangement of mega- and microsporophylls, which seemed to be unusual. The remaining lower portion was sectioned tangentially near the axis. Another radial section was then made, since the structure of the axis was found not to be well preserved in the uppermost portion. Other sections were cut parallel to the longitudinal sections at 4 mm. intervals. In these sections the details of all organs were exposed.

The general arrangement of the parts in this cone is very similar to that described from other strobili of this genus. The stele is surrounded by a well-preserved parenchymatous layer. The cortex is composed of three layers, exceptionally well-preserved tissues. The outer cortex is continuous with that of the pedicel. The sporangium is attached to the adaxial side of the pedicel for almost its entire length. The pedicel does not develop a heel but bends up, becoming the lamina which overlaps several sporophylls (fig. 3*A*). The megasporophylls are found in the lower part of the cone and the microsporophylls in the upper part. The axis tapers gradually toward the upper end, but the shape of the cone does not corre-

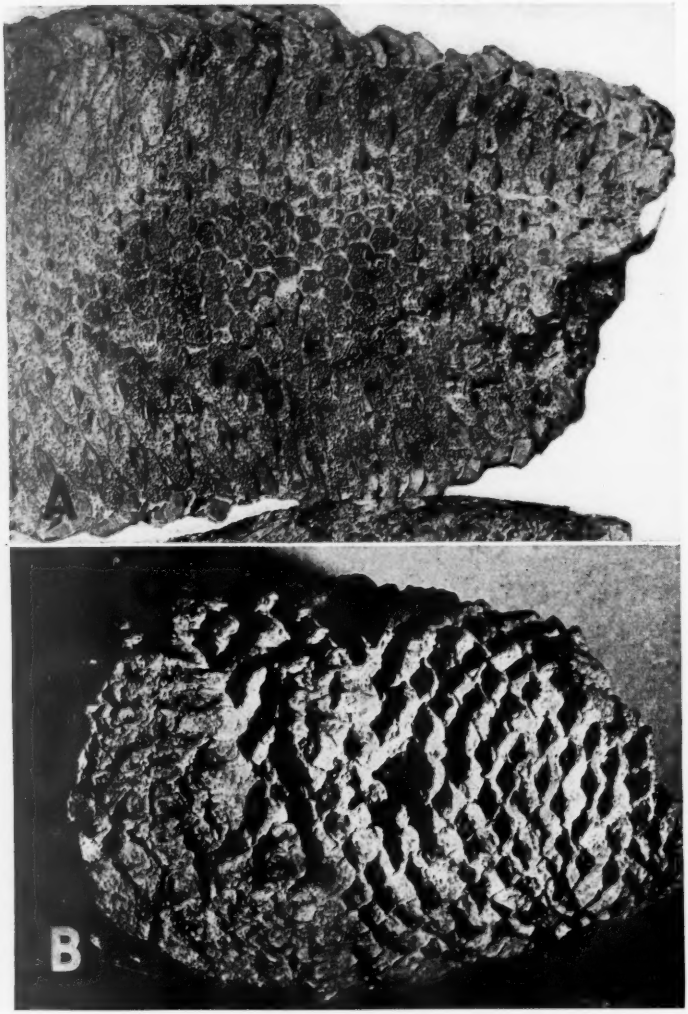


FIG. 3.—*A:* longitudinal tangential section of *Lepidostrobus noei* showing arrangement of megasporophylls and laminae; M3121. *B:* external view.

spond to this feature of the axis. From the axis the conclusion may be drawn that the cone was about 15 cm. long. Thus in the lower two-thirds are megasporophylls with microsporophylls in the upper third.

Cone axis

The central portion of the stele consists of cells which are considered to be undifferentiated xylem elements. Not all are preserved. Those preserved show that they are of the same size and shape—except for the thinner walls and lack of secondary thickenings—as the adjacent metaxylem elements. Owing to the crushing of these cells their exact shape can be determined only by comparison with metaxylem elements. The central cells have no content, are rather long, and their crosswalls are pointed or at right angles to the longitudinal axis. The name pith cannot be applied to this tissue. The xylem is continuous with the central tissue and has a centripetal development, with the protoxylem elements on the outer periphery.

The following measurements are given from the side of the shorter diameter. The metaxylem belt is eight to twelve cells deep, with a total thickness of 300–400 μ. The elements vary from 50 to 130 μ in diameter and have scalariform thickenings. The larger elements are filled with a centrally located substance an average distance of 12 μ from the wall. Elements of less than 25 μ therefore do not have this infiltration. The middle lamella of the cell walls shows up clearly as a black line between the light brown walls. The structure of the stele is exarch, the protoxylem elements being located at the periphery of the stele. These protoxylem elements are smaller, about 20 μ in diameter. The abaxial circumference of the xylem belt is regularly pointed by protuberances of the protoxylem. These are the beginning points of the leaf traces. The protoxylem abuts on a cylinder of parenchymatous cells. It is this layer which in most of the cones described is poorly or not at all preserved. In this cone, however, the preservation is excellent. A camera lucida drawing was made of an area of this parenchymatous region to show all possible details (fig. 4).

The protoxylem is bordered by a zone of two to six tangentially elongated cells measuring about 25 μ in length and 8 μ in width. The walls are thin, the black corners show up sharply, and no intercellular spaces are seen. This zone is interrupted where a leaf trace is to be given off and is several layers in thickness where a leaf trace is to emerge. It resembles the phloem of the leaf traces and represents, by its position and anatomical character, tissue which can be called phloem. Just peripheral to this cylinder is another belt of parenchymatous tissue containing numerous leaf traces. These two parenchymatous cylinders are not clearly delimited from each other. The outer limit of the peripheral layer can be identified by a peculiar layer of cells. They are thin-walled, elongated tangentially, and their radial walls are here and there crushed and zigzag in shape. In connection with the

cells in the pericycle are found one or more cells with thick walls representing pericyclic fibers (fig. 4*pf*). The innermost layer of the cortex consists of cells of larger diameter (40–70 μ) with a central heterogeneous filling. These cells abut peripherally on a trabecular tissue, which is well preserved. This belt measures 400–800 μ in diameter. The elongated cells range in length from 30 to 200 μ, and run in various directions. The cells on the inner border of this belt usually begin with a smaller diameter than that of the adjacent cells, but enlarge from 6 to 30 μ. Even in the same cell the diameter varies, giving this tissue an irregular appearance.

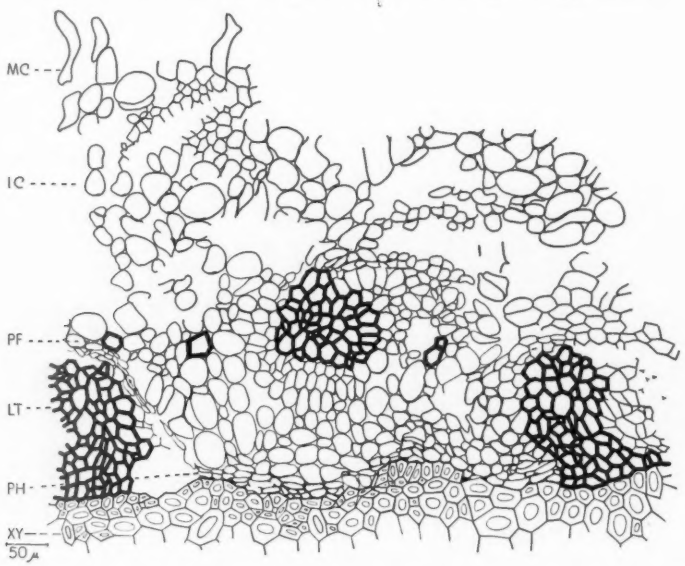


FIG. 4.—Part of axis of *Lepidostrobus noei* (*xy*, xylem; *ph*, phloem; *lt*, leaf trace; *pf*, pericyclic fibers; *ic*, inner cortex; *mc*, middle cortex).

The aerenchymatous nature of this tissue is apparent. The outer cortex starts abruptly and is 1100–1200 μ in thickness. It is made up of two kinds of cells, in two concentric layers. The cells of the inner ring gradually merge into the outer tangential belt. In some instances an irregular black line separates these layers. Among the outer cortex cells of the inner portion intercellular spaces are interspersed, and the diameter of the cells varies a little. On the whole they have a homogeneous content. Although a sharp line cannot be drawn between the peripheral and the central portion, a difference is indicated by the different structure and arrangement of the cells. The cells of the outer portion are arranged in more or less clear radial rows and the diameter decreases peripherally to 23 μ, especially where a pedicel is to leave the axis.

Sporophylls

The sporophylls branch off from the axis at almost right angles, but the inclination of the sporophylls with the axis varies. In the lower portion it is at 70°; at the upper part, at 80°. The sporophylls are arranged in spirals. The phyllotaxy is 2:51. Some indication of this arrangement may be noticed in the regular appearance of the lamina and sporophylls in the long section (fig. 3*A*). The sporophyll is composed of the basal pedicel and the upper sporangium. The pedicel is continuous with the outer cortical tissue of the axis and has cells of the same sclerenchymatous structure. The connective tissue between pedicel and sporangium is in no case preserved, but from the absence of the sporangial wall on the abaxial side of the sporangium it is apparent that the latter was attached to the pedicel for almost its entire length—except for a small distance on the proximal and distal ends. The fragment of the cone represents the lower portion. The lowermost sporophyll is 7 mm. in length, the uppermost 22 mm. When at first the pedicel becomes free from the axis it is triangular in cross section and 2 mm. in the longest diameter. The horizontal diameter is always smaller than the overlapping sporangium, except at the distal end. Here the pedicel enlarges laterally to 5 mm. As the sporangium tapers at the periphery of the cone the pedicel enlarges and becomes rounded on its abaxial side. The cells of the pedicel are of the same structure as those of the outer cortex, isodiametric, sclerenchymatous. The leaf trace runs on the adaxial side. The epidermal cells have thick walls and are arranged in one layer. No difference was noticed between pedicels of mega- and microsporophylls.

Sporangium

The sporangium is attached to the adaxial side of the pedicel. Both mega- and microsporangia are found in *L. noei*. The structure is the same, except the spores. The shape of a sporangium in cross section is shown in figs. 3*A*, 5*B*. It measures 2 × 2.2 mm. By this shape all sporophylls match perfectly. The sporangium is slightly larger than the pedicel, except at the distal end, where the sporangium flattens and tapers tangentially. The connective tissue forms a rather narrow band and is about one-fifth the total width of the pedicel. The walls of both the mega- and microsporangia are made up of typical palisade cells, 25 × 25 μ in size. No difference was noticed in the size of these cells at the corners or on the tip of the sporangia. Inside these wall cells is another layer of jacket tissue made up of elongated cells in which a dark spot (nucleus?) can be seen. In the palisade cells a dark spot is located at the outer periphery (fig. 5*D*). The tapetum centers the jacket cells. It is conspicuous all round the sporangium but is especially well developed at the corners, with a depth of eight to eleven cells. These cells are continuous with the subarchesporial pad. All sporangia are in the mature stage and not yet opened. About the mechanism of dehiscence no peculiar structure could

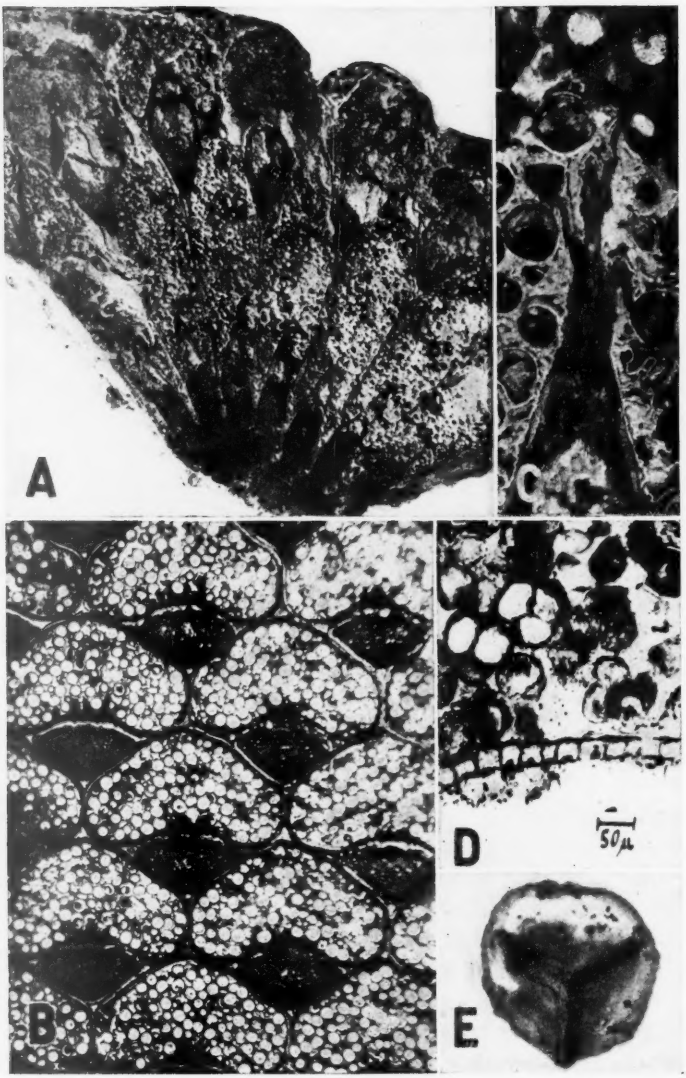
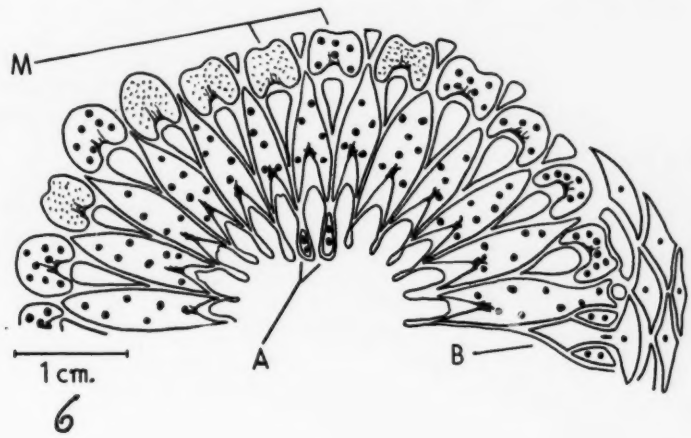


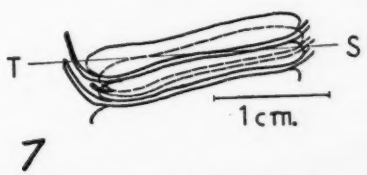
FIG. 5.—*Lepidostrobus noei*. *A:* cross section of cone showing arrangement of micro- and megasporophylls; *B:* tangential section showing megasporophylls; *C:* oblique section through megasporophyll showing sterile tissue plate and megaspores; *D:* microsporangium with microspores in tetrads and sporangial wall; *E:* megaspore.

be noticed in the wall. Sterile plates arise from the subarchesporial pad in both the mega- and microsporangia (fig. 5*C*). In more oblique sections these plates are finger-like projections.

Arrangement of sporophylls

The arrangement of the mega- and microsporophylls is peculiar. Figure 6 represents a drawing from a photograph of a radial section through the upper part of





Figs. 6, 7.—*Lepidostrobus noei*. Fig. 6, arrangement of micro- and megasporophylls. Fig. 7, diagram showing plane of section as given in fig. 6.

the cone. It shows in the peripheral layer thirteen sporophylls—eight megasporophylls and five microsporophylls. The microsporophylls originate separately at three different loci in the same spiral. The median circle represents only megasporangia; the inner circle shows in two instances megasporangia (fig. 6*A*). It would seem that these sporangia are the same as are cut again at the outer circle, which would mean that the same sporangium (*A* and *M*) has megaspores and microspores. From a longitudinal section, however, the solution of this striking feature can be explained in another way. As shown in figure 7, three whorls are

cut longitudinally, of which the upper and lower sporophylls are on the same orthostichy; the median is interlocking. If the upper sporophyll is a microsporophyll and the lower ones are megasporophylls, it is apparent that by a radial section (as indicated by the line *T–S*) the arrangement of sporophylls will be a microsporophyll at the outer circle and a megasporophyll at the median and inner circle. Figure 6 shows various stages of the relation between pedicel and sporophyll. At various points the sporangium overlaps the pedicel, and at *B* the sporophyll is cut in such a way that the pedicel is seen to be continuous with the lamina, while small lateral portions of the overlapping sporangium have been cut off. The pedicels have the same structure in both kinds of sporophylls, although it seems (fig. 6) that the pedicels of the microsporangia are smaller than are those of the megasporangia.

Spores

The megasporangia are filled with numerous megaspores. Some are wholly replaced by pyrites, siliceous compounds, or calcites. After maceration of some megaspores with HCl the thin spore wall remained, which made a detailed study of the spore wall possible. The megaspores measure 270–330 μ and show triradiate markings, each about 120 μ long. The megaspores are separate. Here and there crushed megaspores are seen. The microsporangia are in the upper part of the cone. From a reconstruction on the basis of the preserved axis apparently the upper third of the cone had microsporangia. The microspores are arranged in tetrads (fig. 5*D*). The diameter of a tetrad is 76 μ; of a single spore, about 50 μ in the longer axis. In sections of the microspores often exosporium and endosporium can be seen.

Lamina and ligule

As previously mentioned, the laminae are broken at the point where one would look for the ligule (fig. 3*A*). It was not possible to locate with certainty a structure which might be considered a ligule. The lamina starts with a constriction of the pedicel. It is at that point cylindrical or triangular in cross section, with a diameter of 3.5 – 4 mm. Soon the lamina widens tangentially to 8 mm. On the surface of the cone where the laminae are preserved a small longitudinal median ridge can be seen on each lamina. The longest lamina was 14 mm.

Leaf traces

The leaf traces are well preserved. The starting points are protoxylem elements on the periphery of the stele (fig. 4). The number of such elements is about ten when the leaf trace is freed from the xylem belt. The trace is 100 μ in diameter. The smaller elements are in the center and probably represent the protoxylem. As the leaf trace passes into the parenchymatous layers, the cells adjacent to the bundle are arranged around it like a sheath. In this parenchymatous belt more leaf

traces can be seen than in the regions which follow peripherally. On the abaxial side of the leaf trace a few small, thin-walled cells are separated from the xylem by a layer of parenchymatous cells which represent the beginning of the phloem strand. The leaf trace does not enlarge much as it passes through the trabecular tissue of the middle cortex. In the outer cortex very few leaf traces are seen. Near the middle cortex these traces may be surrounded on all sides with trabecular tissue, although a layer five or more cells in thickness may separate the trace and the middle cortex. A parichnos strand was not noticed in the peripheral part of the cortex or the pedicel. At the entrance of the leaf trace into the pedicel the diameter of the trace is not increased. When the trace bends in the outer cortex to enter the pedicel, the arrangement of the cells outside the bundle on the phloem side is changed. The sclerenchymatous cells on the adaxial side run in their ordinary straight way and simply abut on the bundle sheath; the cell on the abaxial side bend to enter the pedicel. Just at this point parenchymatous cells parallel to the main axis are seen which connect the leaf trace with sclerenchymatous cells. This layer is about 300 μ in thickness. In the pedicel the leaf trace runs on the adaxial side, phloem downward. It is larger than in the axis and has a radial diameter of 200 $\times$ 500 μ, of which the latter means the axis in the xylem-phloem direction. In the lamina the leaf trace takes a central position, is of large diameter (400 $\times$ 500 μ), and collateral. The epidermis is composed of a layer of thick-walled cells.

Discussion

A comparison of *Lepidostrobus coulteri* and *L. noei* with the already described species of *Lepidostrobus* does not reveal many striking peculiarities in these new cones, but they appear to be good distinct species.

In size the cones agree with *L. brownii* (**17**), which has a diameter of 5–7.5 cm., and with *L. kentuckiensis* (**13**) and *L. bertrandi* (**16**), which have diameters of 5 cm. Data of the actual length of the cones are not always available, for generally only fragments are known. The longest in this group is *L. coulteri*, with an actual length of 22 cm. (**5**).

The axis varies much in size and structure in this genus. The ratio between the diameter of cone and axis is not constant: in *L. brownii* (**17**) it has a range of from four to ten, in *L. coulteri* and *L. delagei* (**17**) about seven, in *L. oldhamius* (**11**) five to ten, in *L. noei* about ten, in *L. bertrandi* (**16**) four, in *L. kentuckiensis* (**13**) five, and in *L. binneyanus* (**1**) three. In *L. masleni* (**3**) the axis decreases from 1.28 mm. at the base of the cone to 0.53 mm. at the top. The diameter decreases in the same ratio. This is not true in *L. noei*.

According to the structure of the stele the cones may be placed into three groups. To the first group belong the protostelic forms with xylem throughout, such as *L. schimperi* (**17**). In a second group can be placed those forms which have

not developed a special pith tissue but have central undifferentiated embryonic tissue. Here belong the forms described as having "fibres primitives," as *L. brownii* (17), *L. kentuckiensis* (13), and *L. bertrandi* (16). In this group fit also *L. coulteri* and *L. noei*. To the third group belong the forms with a central pith—the siphonostelic cones, as *L. binneyanus* (1), *L. masleni* (3), and *L. arberi* (1). From the phylogenetic standpoint, considering only this character, the first group probably is the most primitive while the last one with a pith is most advanced. The forms in the second group bridge the other two groups.

In the central embryonic cells of *L. coulteri* and *L. noei* are found thin walls and walls with thin scalariform thickenings, pointed cells with oblique cross-walls, and cells with end walls at right angles. In all forms the scalariform elements are central, while spiral or annular thickenings are found in the peripheral protoxylem elements. The nature of the region just outside the xylem cylinder is much disputed. This is due not only to insufficient preservation in most forms, but partly to a real difference in the arrangement of tissues in different cones and partly to a lack of uniformity of nomenclature. The tissue surrounding the xylem belt was probably of delicate nature. If it did not decay it became crushed and only in a few cases is it well preserved. These small cells of a parenchymatous nature were called cambial cells in *Lepidodendron* by WEISS. SEWARD and CAMPOS (3) consider them meristematic. BERTRAND described them as "fibres primitives" and considered them related to phloem cells. MASLEN (11) speaks of them simply as parenchymatous cells. BOWER (2) did not recognize any phloem, although the specimen he described was well preserved. SCOTT (15) calls them phloem cells. In *L. noei* the tissue (fig. 4*ph*) consists at certain points of several layers. The name phloem seems to be appropriate. Hypertrophied cells with darker contents are described by BERTRAND and SEWARD as secretory cells. Polygonal thick-walled cells interspersed at regular intervals are figured by BOWER (2). They have not been recorded from other cones, and according to position and anatomy they are described as pericyclic fibers. Barred cells, occurring on the outer periphery of the xylem, have been observed in *L. oldhamius* (11) and more rarely in *L. bertrandi* (16) but have not been noticed in the other forms here mentioned.

The endodermis, which in recent pteridophytes is markedly differentiated from the other cortical and pericyclic layers, was described and figured by BOWER (2) and ZEILLER (17) for *L. brownii*. BOWER recognized an endodermis "by the more sharp definition of its walls . . . . and the radial walls with characteristic dots" (2). The endodermis BOWER observed was not continuous. ZEILLER indicated tentatively as endodermis a cell layer characterized by distorted zigzag radial walls. He could not follow this sheath around because in many instances the

tissue was decayed (**17,** pl. II, fig. 8). This wall deformation was the only anatomical character; no wall thickenings or dots were noticed.

Division of the cortex into three parts is in general use. The inner cortex is usually preserved, but if not, the beginning of the internal periphery can be made out by the abaxial projections of the leaf trace, as is seen also in *L. bertrandi* (**16**) and *L. oldhamius* (**1**). The nature of the middle cortex as an aerenchymatous tissue, as in *Selaginella*, is well shown in *L. noei*. The lacunar tissue is probably easier attacked and destroyed by decay organisms than the other tissues. The tissue of the middle cortex is continuous into the leaves and laminae as a small strand on the phloem side of the bundle, the parichnos. In different species the development of the parichnos shows in various degrees. In *L. coulteri*, as in other species, it branches at the distal end of the pedicel and accompanies the leaf trace on both sides for a certain distance; in *L. brownii* (**17**) it terminates in the outer cortex; in *L. noei* almost the same is true; in other forms, as *L. bertrandi* (**16**), no parichnos is seen at all. The presence of the parichnos seems to be specific. It is not impossible that the degree of its development may reflect the ecological habitat of the specimen.

The sporangia are usually the shape of a five-sided polygon. The sporangium is attached basally to the adaxial side of the pedicel in most forms, or it is median —as in *L. masleni* (**3**)—at a distance of 1 cm. from the base of the pedicel. The sporangia of *L. noei* are quite long, reaching 20 mm. and being exceeded only by those of *L. masleni* (**3**), which attain a length of 27 mm. The jacket layer, consisting of an inner and an outer palisade layer, is well differentiated from the tapetal layer in *L. noei*. Owing to early development, the subarchesporial pad extends farther into the sporangium, but later becomes appressed to the floor of the latter, as can be seen by the compressed cells. Probably the growing spores are responsible for this.

Sterile tissue plates have been noticed in *L. oldhamius* and *L. masleni* (in the microsporangia), and their structure is specific. Scott (**15,** Vol. II, p. 165) mentions evidence of sterile tissue in megasporangia but did not illustrate his statement. *L. noei* shows sterile tissue plates also in the megasporangia (fig. 5*C*). Special enlarged palisade cells at certain points have been described in several forms (such as *L. brownii* and *L. coulteri*). About the function there is no agreement. Two species are known in which the number of megaspores in a sporangium is greater than sixty-four, *L. brownii* (**17**) and *L. noei*. A comparison of the size of micro- and megaspores within a species shows that the ratio in the homosporous species is 1:1, but varies in the heterosporous species. In *L. bertrandi* it is 1:2; in *L. noei*, 1:5; in *L. brownii*, 1:10; in *L. scottii*, 1:30–40, and in *L. masleni*, 1:45. Phylogenetically *L. masleni* would be considered in this respect highly specialized

and *L. bertrandi* less advanced. Thus a cone may be highly specialized in one respect and primitive in another. The presence of the heel seems to be specific. It is present in most forms but is lacking in *L. noei*. The presence of a ligule is specific for *Lepidostrobus*. It may be inserted in a ligular pit as in *L. arberi* (1) or occur without a ligular chamber.

The structure of the leaf trace is the same in various forms. It is collateral except in *L. oldhamius* (1), where it appears to be concentric at the apex of the sporophyll. The number of xylem elements in the leaf trace varies among the species and may range from nine to as many as seventy. In traversing the axial tissues the leaf trace is accompanied in a peripheral direction by each of the tissue systems through which it passes; for example, a trace passing through the middle cortex is accompanied by phloem, parenchyma, and inner cortical tissue in the immediately peripheral layer. Thus xylem, phloem, parenchyma, etc. are found displaced in a peripheral direction immediately adjacent to the leaf trace.

The sporophylls are arranged in circles or spirals. The data given by ZEILLER (17) show that the phyllotaxy is not constant in *L. brownii*, the number of orthostichies varying from seventeen to thirty-three. In *L. noei* the number is even larger, fifty-one. The partly irregular arrangement of mega- and microsporophylls in *L. noei* seems to be unique. Only by study of additional heterosporous forms can it be determined whether this arrangement of sporophylls is common.

The epidermis is composed usually of thick-walled cells. Some species show a special hypodermal layer.

Summary

1. Two species of *Lepidostrobus* are described, *L. coulteri* Jongmans and a new species, *L. noei* Mathews from Kentucky. Two specimens of the former are described in detail, since nothing was known previously about the structure of the axis of that species. A description and several diagnoses are made on the basis of sections. The size, the presence of a heel, the extension of the leaf traces high into the lamina, together with a well-developed parichnos seem to distinguish these specimens from other described cones. The five cones in the collection suggest a classification among homosporous Lepidostrobi.

2. *L. noei*, sp. nov., is unique in that it shows many features not yet observed in other forms. The middle cortex shows the trabeculate structure quite well, although the parichnos does not extend beyond the central portion of the outer cortex. The heel is absent. The cone is heterosporous, the microsporophylls occupying the upper one-third of the cone and the megasporophylls the lower two-thirds. No difference in structure was noticed between micro- and megasporophylls. The ratio in size of the spores is 1:5. The arrangement of micro- and megasporophylls in the same whorl is unusual.

3. A comparison with the hitherto described cones has been made and differences and similarities pointed out. Phylogenetic considerations have been contrasted with the geological appearance of the cones.

The writer is indebted to the late Dr. A. C. Noé for suggesting this problem and supplying the material.

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PEPTIDASE ACTIVITY IN THE AVENA COLEOPTILE PHYTOHORMONE TEST OBJECT

GEORGE S. AVERY, JR., AND K. LINDERSTRØM-LANG

(WITH NINE FIGURES)

Introduction

In a previous investigation on the distribution of enzymes in plant tissue, peptidase activity was determined throughout the length of the *Hordeum* coleoptile and its inclosed first foliage leaf. The ratio between peptidase and a quantity which presumably was proportional to the protein content in the tissue was found to be fairly constant throughout the entire length of the coleoptile plus the inclosed first foliage leaf (**5**).

The object of the present study was to determine the distribution of peptidase throughout the *Avena* coleoptile (without its inclosed first foliage leaf) at different growth stages, both in relation to "reduced weight" of the tissue and in relation to number of cells. This particular material was chosen since the cellular morphogenesis of *Avena* is now well understood. Moreover, the coleoptile presents many interesting features in relation both to growth problems and to specific plant hormone problems.

In the germinating seedling of *Avena* it has been shown (**2**) that cell division in the embryonic coleoptile starts within 24 hours after the seeds are placed under suitable conditions for germination. Cell divisions are more numerous during early germination but continue with appreciable though diminishing intensity until the coleoptile is approximately 10 mm. in length (**1**). As long as cell division persists it goes on with equal intensity throughout the coleoptile. The coleoptile grows in length only, not in diameter (that is, growth is polarized). After a length of 10 mm. has been reached, further elongation is due almost entirely to extension of existing cells. Toward the end of the period of growth by cell extension (near maturity), the zone immediately proximal to the coleoptile tip undergoes proportionately greater extension than other portions of the coleoptile.

Thus the coleoptile is an organ in which the major period of cell division is limited to its early development and the major period of cell elongation is limited to its later development. Cells in the distal 0.5 mm. neither divide nor elongate as the coleoptile grows. They are essentially isodiametric, with large nuclei, dense cytoplasm, and small vacuoles (**6**). Proximal to this terminal zone, cells elongate considerably during growth of the coleoptile, and become highly vacuolate. It is this difference between the terminal cells and those proximal to them which pro-

vides the most obvious cytological basis for a physiological gradient from tip to base of the coleoptile.

The presence of an auxin gradient in the coleoptile, with a high concentration at the tip and a gradually decreasing concentration toward the base (**8, 7**), cannot be correlated with the synthesis of new protoplasm because there is no distinct embryonic growth center in the coleoptile at any time in its development. Hence such a gradient might be attributed to: (*a*) the presence of hormone-secreting cells at the tip or (*b*) the movement from the cotyledon or endosperm of the auxin or its "precursor" through the vascular bundles (**3**) to the tip, from which point it could be dispersed into the nonvascular tissues. In any case, the question which immediately arises is: to what extent does this auxin (perhaps more than one "kind") influence the metabolism of the tissues of the coleoptile?

Investigation (methods)

PREPARATION OF MATERIAL AND GENERAL METHOD.—Seeds of *Avena sativa* L. var. Victory (Svalöf) were carefully selected and weighed for uniformity; only those between 31 and 33 mm. were used. They were soaked 1–3 hours at laboratory temperature, then placed in preparation dishes on thoroughly moistened filter paper. The dishes were placed in a dark chamber, at 25° C., and supported at a 60° angle so that the coleoptiles could grow upright without bending or coming in contact with the glass walls of the dish (fig. 1). Enough seeds were started each day so that at all times there were seedlings with coleoptiles 1.5, 4, 10, 17, and 36 mm. in length. These lengths were selected because of our knowledge of cell behavior at these lengths in the same variety (**1**).

When the coleoptile of a seedling had reached the desired length it was removed from the dark chamber, carefully cut away from the seedling (below the level of its attachment to the embryonic axis), and imbedded in 45° paraffin, after which it was promptly placed and kept in ice water until sectioned at 125 μ or 250 μ on a rotary microtome. Special adjustment of the microtome was necessary to cut uniformly at these thicknesses. Never more than 1 hour elapsed between the time of imbedding and completion of the cutting. Care was taken to prevent the segments from coming into contact with anything. To do this the paraffin ribbon with the coleoptile segments in it was transferred every three to five cuts to a moist chamber, where it was placed on a glass slide. One edge of the paraffin ribbon was all that touched the slide; the portion containing the segments projected over the edge. Upon completion of cutting, the coleoptile segments with surrounding paraffin were removed one by one from the moist chamber to the edge of a slide on the stage of a binocular microscope, the paraffin of the ribbon again acting as a support to keep the segment from touching anything. First the embryonic foliage leaf was removed from the central cavity, then each segment was quickly

lifted from the paraffin with the aid of a small hooked scalpel and placed in a standard volume of 30 per cent glycerin in a 250-cmm. vessel. The total length of time elapsing between sectioning the coleoptile and placing the segments in glycerin was 20–40 minutes, except when unusually large numbers of segments were

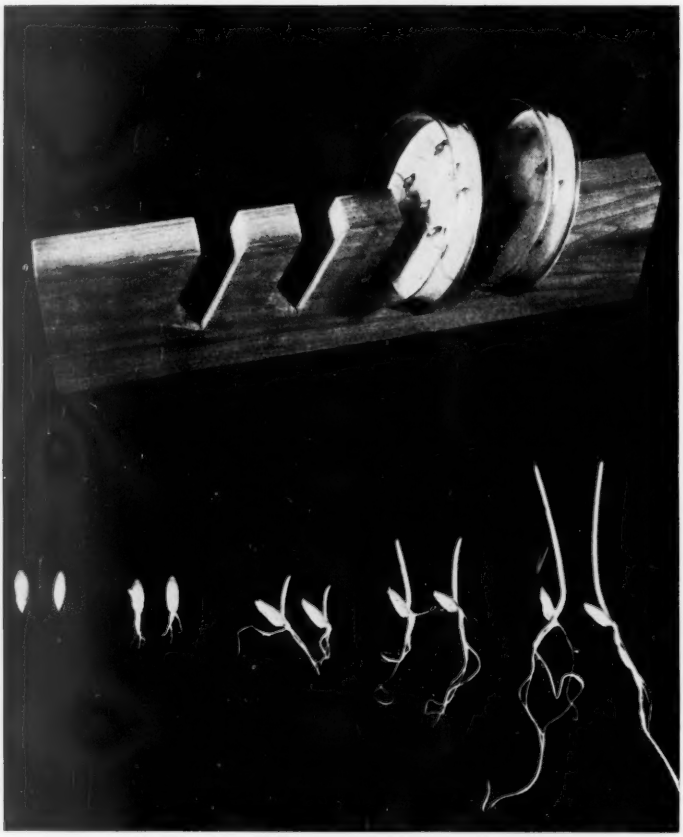

FIG. 1.—Above: Method of culturing *Avena* seedlings. Petri dishes held in nearly upright position for straight growth. Below: Seedlings in various stages of development (grown in darkness at 25° C.): 1.5 mm., 1 day old; 4 mm., 2 days old; 10 mm., 3 days; 17 mm., 4 days; 36 mm., 5 days old.

cut from the longer coleoptiles. Tests in which an additional 30 minutes to 1 hour elapsed showed no discernible differences. Peptidase determinations were made according to the method of LINDERSTRØM-LANG and HOLTER (4). Immediately upon completion of the placing of segments in glycerin, the substrate alanylygly-

cine (0.2M) was added with a semi-automatic pipette; and after stirring electromagnetically, the vessels were placed in an incubator at 40° C. for 4 hours, for splitting of the dipeptide to occur. (Preliminary tests showed this length of time to give good splitting with the amount of tissue involved.) At the end of the 4-hour

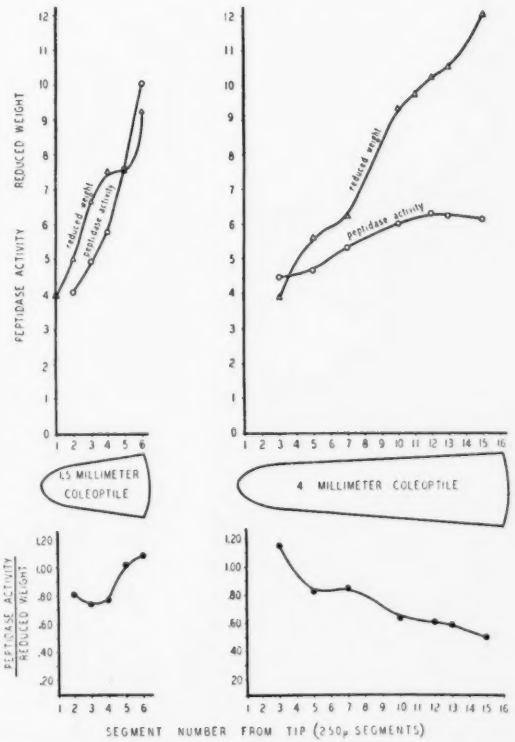

FIG. 2.—Upper left: Reduced weight and peptidase activity of successive 250-μ segments from tip to base of 1.5-mm. coleoptile, also (lower left) peptidase activity per unit weight in various portions of the coleoptile, calculated from preceding. Upper and lower right: Same, but for 4-mm. coleoptile (portion of tip extending beyond ordinate in this and other graphs was in all instances less than a full section of 250-μ thickness, hence not tested).

period, splitting was stopped by the addition of 0.05N hydrochloric acid in 95 per cent alcohol, added with an automatic pipette. To this was added approximately 150 cmm. of an acetone solution of naphthyl red, which was used as an indicator. Titration was completed by adding known amounts of hydrochloric acid in alcohol with a microburette. Cleavage of the substrate was determined in cmm. of hydrochloric acid necessary to complete the titration. Numerous segments were used for

controls. The results are expressed in arbitrary peptidase units: the increase in number of amino groups expressed as cmm. 0.05N hydrochloric acid.

THICKNESS OF SEGMENTS.—In order to make certain that under the experimental conditions there was sufficient contact between enzyme and substrate, a

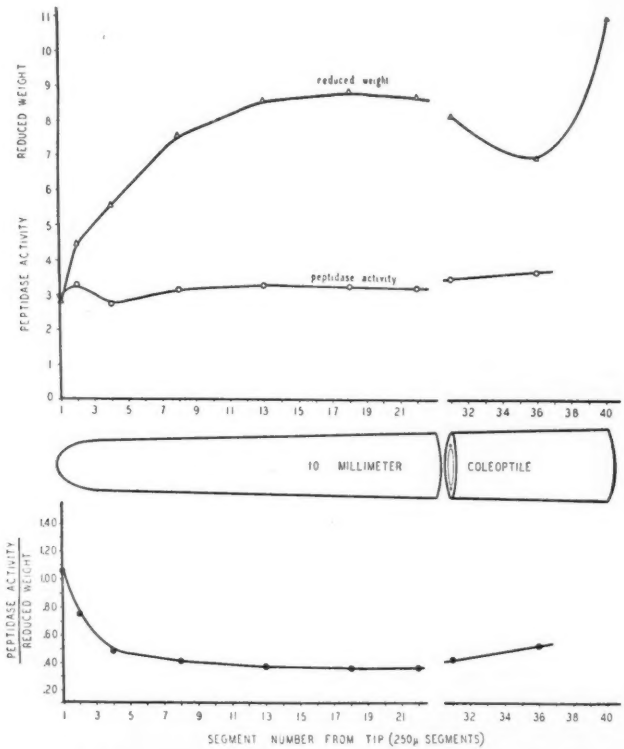

FIG. 3.—Above: Reduced weight of 250-μ segments at various levels between tip and base of 10-mm. coleoptile, also peptidase activity of corresponding segments from another 10-mm. coleoptile. Below: Peptidase activity per unit weight in various portions of coleoptile, calculated from preceding.

3.7-mm. coleoptile was cut into serial segments as follows: one segment 250 μ in thickness; two segments 125 μ in thickness; one segment 250 μ; two segments 125 μ; etc. The two 125-μ segments were placed in a single vessel so that alternate vessels contained a single 250-μ segment and two 125-μ segments. The results, when plotted, gave a smooth curve throughout the length of the coleoptile, indicating that both 125-μ and 250-μ segments completely liberated the enzyme.

WEIGHING OF SEGMENTS.—The imbedding and cutting procedures were used as

given, and segments from various portions of the coleoptile were placed in the gradient tube for determining "reduced weight" (figs. 2–7), according to an unpublished method of LINDERSTRØM-LANG. The so-called reduced weight represents

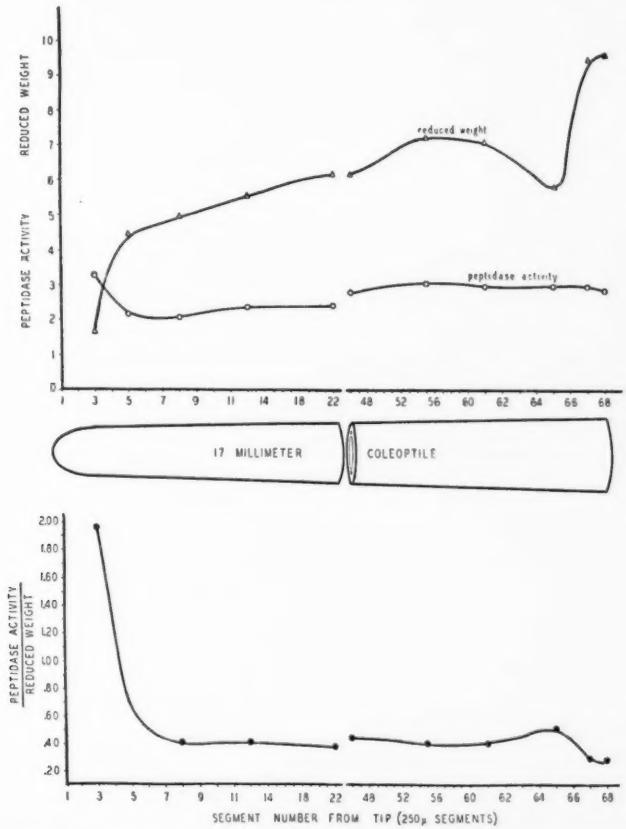

FIG. 4.—Continuation of fig. 3, but for 17-mm. coleoptile (changing scales on abscissae are of no significance; all segments for given coleoptile are of same thickness).

the solid matter of the cells; the weight of the water in the tissue therefore is not included.

DETERMINATION OF CELL NUMBER.—A number of representative coleoptiles were selected at the 4- and 10-mm. stages of growth, fixed in Navashin's fluid, and following the usual steps of dehydration and paraffin infiltration were imbedded and cut in serial transections at 25 μ; other serial longisections were cut at 20 μ.

Cell counts were made of the total number of cells appearing in each successive 25-μ transection, and the average for each five sections was then determined by the following procedure: (*a*) from median longisections the average cell length in microns was determined for cells in the distal 125 μ, then for the second 125-μ portion, the third, etc., until the average cell length for each 125-μ segment of the entire coleoptile was known; (*b*) cell counts were then made for each 25-μ transection in the terminal five sections, totaling 125 μ, and these were averaged.[1] The average transection cell count was then multiplied by 125 to give the total length

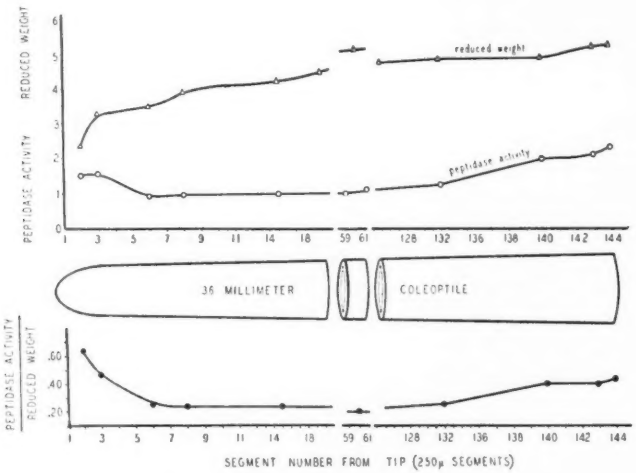

FIG. 5.—Continuation of previous figures, but for 36-mm. coleoptile

(in microns) of all cells in that particular segment, and this sum was divided by the average length of cells in the segment to give the total number of cells in that particular segment. The results of such determinations for single coleoptiles at the 4- and 10-mm. stages of development appear in figures 8 and 9.[2]

Investigation (results)

1.5-MILLIMETER COLEOPTILES.—Coleoptiles 1.4–1.6 mm. were prepared as indicated, cut into 250-μ segments, and peptidase determinations made on successive sections. There were slight differences in the magnitude of enzyme activity

[1] Vascular bundles not included; in a 4-mm. coleoptile, at the proximal end of the tip 125-μ segment, the bundles constitute 0 per cent of the area of a transection of the coleoptile; at the base of the third segment, 3.4 per cent of the area; fifth segment, 5 per cent of the area; tenth segment, 8.7 per cent; and fifteenth segment, 4.8 per cent.

[2] A statistical study of cell number in the coleoptile is now in progress.

in different coleoptiles, but in all instances the distribution of the enzyme agreed with data given for the 1.5-mm. coleoptile (fig. 2, upper left). With the exception of the tip-most segment, peptidase activity in 1.5-mm. coleoptiles was higher throughout than in any of the older coleoptiles.

On the basis of the reduced weights determined for a 1.6-mm. coleoptile, the peptidase activity per unit weight decreases from base to tip (fig. 2, lower left). At this stage of development, cells throughout the coleoptile are undergoing more rapid cell division, and therefore more rapid synthesis of protoplasm, than at any later stage in their growth.

4-MILLIMETER COLEOPTILES.—At this stage of coleoptile growth peptidase activity was still high throughout (fig. 2, upper right), but except for the tip-most section the activity is not so great as in the 1.5-mm. coleoptiles. Reduced weights of successive segments from tip to base of a 4.1-mm. coleoptile are given in figure 2 also, and on the basis of these determinations the peptidase activity per unit weight (fig. 2, lower right) is highest at the tip and decreases steadily toward the base. This is the reverse of the gradient noted in coleoptiles 1.5 mm. in length.

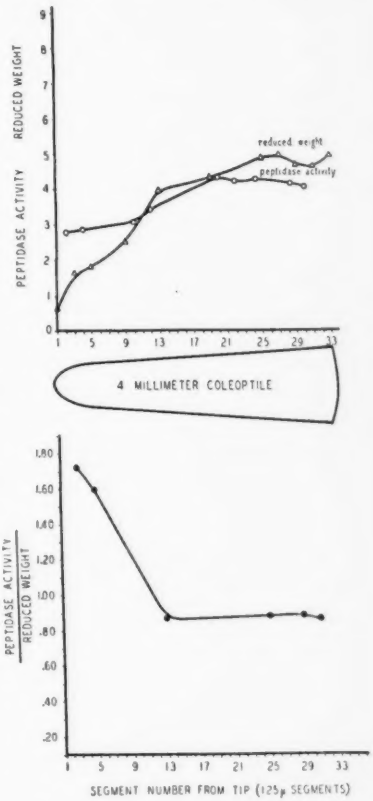

FIG. 6.—Above: Coleoptile 4 mm. in length, cut into 125-μ segments. Alternate segments used for determining reduced weights, and peptidase activity. Below: Peptidase activity per unit weight in various portions of coleoptile, calculated from preceding.

10-, 17-, and 36-MILLIMETER COLEOPTILES.—Peptidase activity and reduced weights of selected segments from coleoptiles of these lengths are given in figures 3–5. In each instance the gaps in the abscissa indicate that for these segments enzyme activity was not determined. Reduced weights were obtained at equal distances throughout the greater part of the length of the coleoptile, and even though omitted from the graphs at the gaps indicated, the general trend may be observed. The higher reduced weights of segments at or near the base of the 10- and 17-mm. coleoptiles agree with the determinations for younger coleoptiles, but

just above the basal segments weight decreases somewhat, only to rise again through the middle portion of the organ. Reduced weights of selected segments from the 36-mm. coleoptile show a very gradual rise from tip toward base, with but slightly increased weight through the middle of the coleoptile.

In general, the reduced weight of segments throughout the length of the coleoptile decreases with increasing age. Peptidase activity also decreases as the age of the coleoptile increases, but is always greater at the tip and base of the

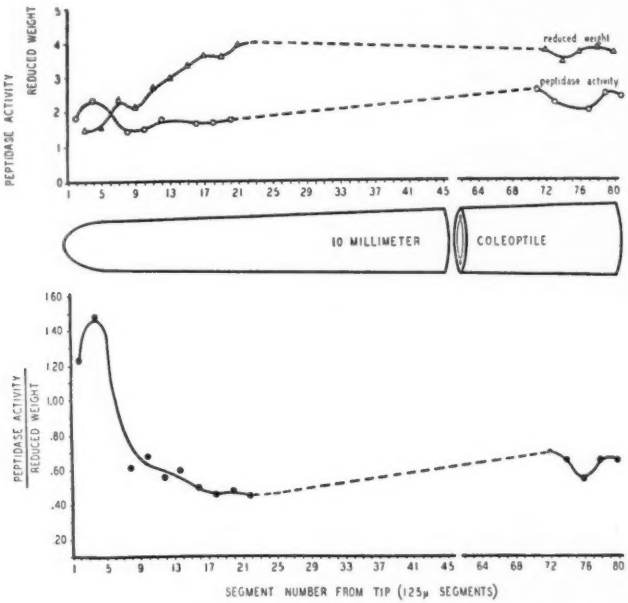

FIG. 7.—Continuation of fig. 6, but for 10-mm. coleoptile

coleoptile than through the middle portion. Peptidase activity per unit weight is much greater in segments near the tip, particularly in the 10- and 17-mm. coleoptiles.

4- AND 10-MILLIMETER COLEOPTILES, ALTERNATE SEGMENTS WEIGHED AND PEPTIDASE ACTIVITY DETERMINED.—In all the foregoing experiments, reduced weights and peptidase activity were determined on different coleoptiles, but of the same size for each age group. In this experiment a given coleoptile was cut into 125-μ segments. Alternate segments were used for determining reduced weights, the remaining ones for determining peptidase activity. Hence peptidase activity per unit weight was obtained for segments throughout the length of a

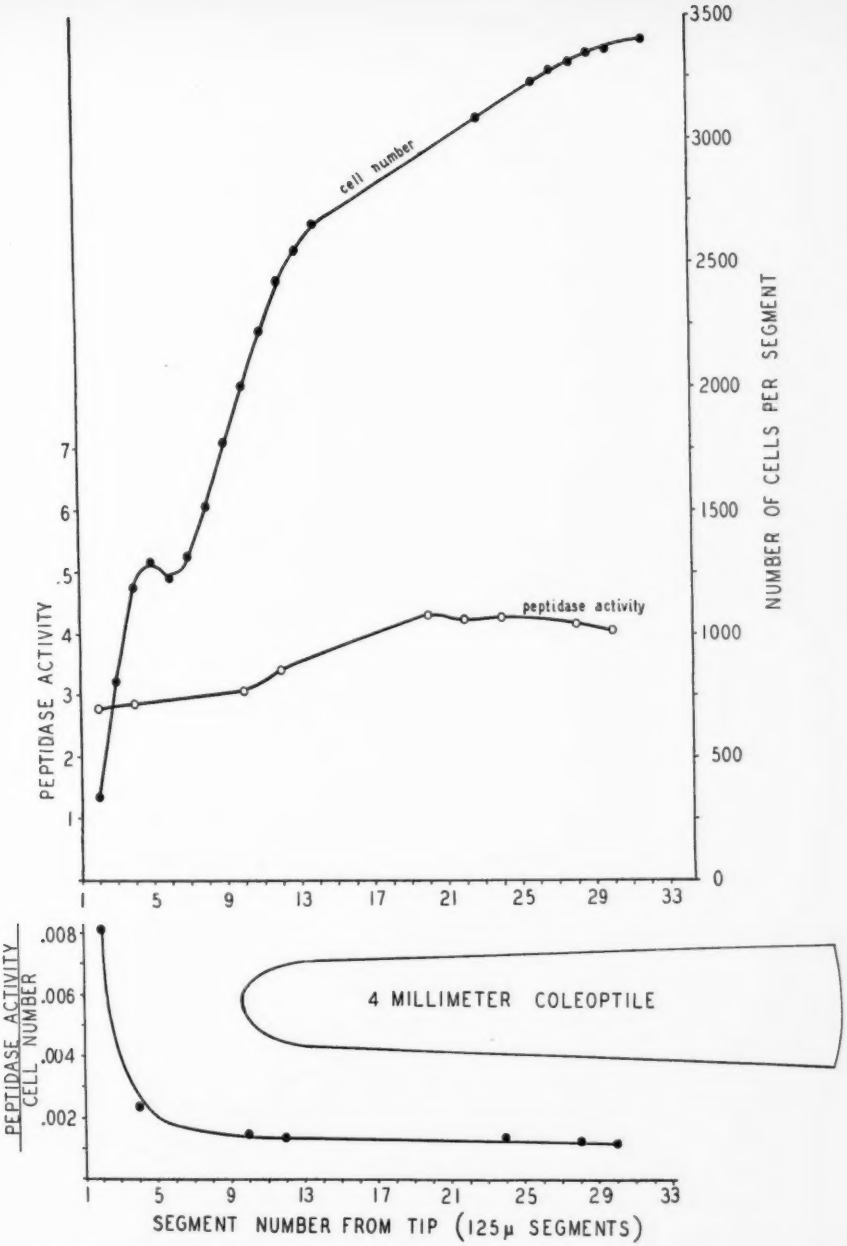

FIG. 8.—Above: Peptidase activity same as in fig. 6, but including number of cells per 125-μ segment (vascular cells omitted, but these occupy only *ca.* 5%, on average, of cross-sectional area). Below: Peptidase activity per cell in various portions of coleoptile, calculated from preceding.

given coleoptile (figs. 6, 7). The results corroborate those reported, and bring out even more sharply the fact that peptidase activity per unit weight of tissue is much greater at the tip of the coleoptile.

PEPTIDASE ACTIVITY IN RELATION TO NUMBER OF CELLS PER SEGMENT.—Figure 8 (above) shows that the number of cells per 125-μ segment increases considerably—

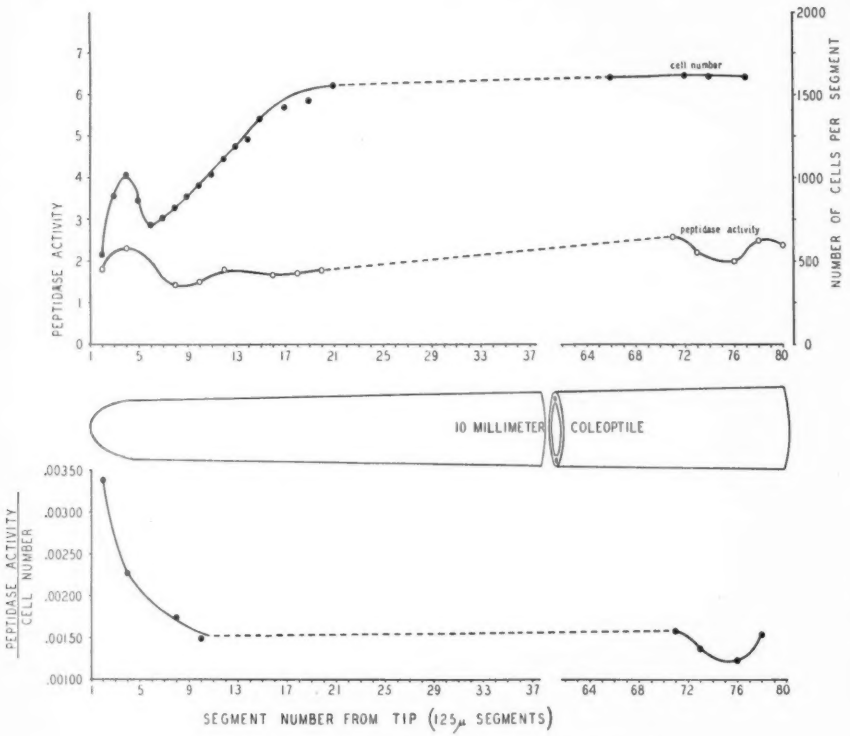

FIG. 9.—Continuation of fig. 8, but for 10-mm. coleoptile

more than tenfold—from tip to base in a 4-mm. coleoptile. Peptidase activity, on the other hand, is only slightly greater in segments from the middle and basal portions of a 4-mm. coleoptile. On a per cell basis, this means that the peptidase activity is considerably higher at the tip than in other portions of the coleoptile (fig. 8, below).

Cell number per segment for a 10-mm. coleoptile is given in figure 9 (above). While the total number of cells in the whole coleoptile is much greater than in one

4 mm. in length, there are fewer cells per segment because the average cell is approximately twice as long. Peptidase activity per cell is here again much higher at the tip than in other portions (fig. 9, below).

The "pore" of the coleoptile occurs in the fourth segment; around it the cells are somewhat smaller. Distal to the pore there is little or no cell elongation as the coleoptile grows, but cells proximal to it stretch appreciably. This accounts for the jog in the curve (fig. 9, above).

AUXIN IN COLEOPTILES.—Tips were removed from coleoptiles of the same age groups as the preceding and their auxin content tested by immediately placing them unilaterally on decapitated test coleoptiles for 2 hours, according to the method of WENT. Each figure represents the average of forty-eight coleoptile tips, from coleoptiles of the lengths indicated.

COLEOPTILE LENGTH

	1.5 MM	3 MM	4 MM	10 MM	17 MM	36 MM
Auxin concentration (in degrees *Avena* curvature)	2.1	4.8	6.2	9.9	9.9	8.9

It is clear that there is an increase in auxin concentration in tips of coleoptiles between 1.5 and 10 mm. in length; there is no further increase in the 10–17-mm. stages; and there is a slight decrease as the coleoptile nears maturity (36 mm.).

As for the distribution of auxin in coleoptiles, THIMANN (**8**) (see also SÖDING, **7**), working with coleoptiles 25 mm. in length, showed the presence of an auxin gradient from tip to base; highest concentrations were present at the tip, with decreasing amounts at successively lower levels. The higher concentration of auxin in the tips is correlated with higher peptidase activity per unit weight at the tip; also higher peptidase activity per cell.

Discussion

That the distal ends of coleoptiles 1.5 mm. in length were found low in auxin, by the method used, cannot be shown at this time to have definite bearing on the fact that peptidase is relatively low in the tips of such coleoptiles. Nor at this time can the higher auxin content of the tips of 4-mm. and older coleoptiles be causally related to the higher peptidase activity per unit weight of tissue, or per cell. These points take on added interest, however, in the light of the approximate parallelism of the peptidase and auxin gradients in older coleoptiles. Whether auxin in higher concentrations can effectively act as an enzyme activator remains to be demonstrated, but suggestive evidence is at hand.

Physiologically active substances other than auxin also have been demonstrated

to be present in higher concentrations in the tip than elsewhere in the coleoptile, for example, ascorbic acid and vitamin B_1.

The most important fact on which to base an explanation of the high peptidase activity of the coleoptile tip is the character of the cells in the terminal 0.5 mm. They differ sharply from cells proximal to them; as the coleoptile grows they undergo no division and very little enlargement. As a result they are richer in protoplasm, that is, they have large nuclei and dense cytoplasm. Because of this obvious protoplasmic difference we feel that an investigation of the ratio of peptidase activity to nitrogen content in the tip cells is called for, and such work will soon be under way. The relation between such histological differences, which undoubtedly find their chemical images in peptidase distribution and production or transport of auxin in the coleoptile tip, is more obscure.

Summary

1. *Avena* seedlings were grown in darkness at 25° C. on moist filter paper in preparation dishes. When the coleoptiles were 1.5, 4, 10, 17, and 36 mm. in length, they were uniformly sectioned at 125 or 250 μ on a rotary microtome. Peptidase determinations were made on these segments, and their reduced weights were obtained by use of the gradient tube. Cell counts were made for successive 125-μ segments of coleoptiles 4 and 10 mm. in length. On a per segment basis, peptidase activity, reduced weight, and cell number were found to decrease in progressively older coleoptiles.

2. For any given coleoptile 4 mm. or more in length, enzyme activity per unit weight of tissue, or per cell, was consistently greater at the tip. Correlations between morphological structure and auxin and peptidase gradients in the coleoptile are pointed out.

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AGGLUTINATION TEST APPLIED TO STRAINS OF PHYTOMONAS STEWARTI

GEORGE L. MCNEW AND ARMIN C. BRAUN[1]

Introduction

Strains of the sweet-corn wilt bacterium, *Phytomonas stewarti* (E.F.S.) Bergey *et al.*, that differ in virulence, colony characteristics, color, and physiological abilities have been described in recent years (**8, 13, 14, 15, 24, 27**). The bacteria from wilted plants in different sections of the United States have been classified into three main types by IVANOFF *et al.* (**7**), but no attempt has been made to determine the relationship of these and other strains. Studies were undertaken, therefore, to determine whether some of these strains differed in their serological properties, and if so, what their relationships to one another might be. This paper reports the results of cross agglutination tests made with strains from different geographical areas and with variant types obtained from some of the strains. Data are also presented on the agglutination of ninety field isolates of *P. stewarti* and of several other species of wilt bacteria.

Material and methods

Fifteen strains of *P. stewarti* were used as antigens for producing immune sera. These strains were selected because they represented different geographical areas as well as different cultural and pathogenic types. Their origin and distinctive characteristics were as follows:

B–11.—A highly virulent strain isolated at Princeton, New Jersey, in September, 1935 (**14**). The culture had lost some of its virulence during the 3 years it had been maintained on nutrient-dextrose agar.

B–1011a.—An almost avirulent single-colony isolate obtained from B–1011 (**14**), which was a highly virulent strain obtained from B–11.

B–1311.—A virulent single-colony isolate obtained from B–1011. At one time (**15**) this strain was the most virulent of all progeny obtained from strain B–11, but it had lost some of its virulence at the time of these experiments.

B–1611.—An avirulent single-colony isolate obtained from B–11 in July, 1936.

B–1111.—An almost avirulent single-colony isolate obtained from B–11. This culture represents a type that is weakly virulent, apparently because of its inability to use inorganic nitrogen (**14**).

[1] The writers are indebted to Drs. CHARLOTTE ELLIOTT, E. J. WELLHAUSEN, R. E. LINCOLN, W. H. BURKHOLDER, G. L. PELTIER, J. G. HARRAR, and H. H. THORNBERRY for supplying some of the cultures used.

B–102.—A highly virulent strain isolated from a naturally infected resistant inbred line of maize (6–215) at Princeton, in August, 1928.

B–103.—A highly virulent strain isolated from an extremely susceptible inbred line (C–18) at Princeton, in August, 1938.

B–107.—A highly virulent strain isolated from maize by Dr. ELLIOTT at Orizaba, Mexico, in December, 1938.

B–108.—A highly virulent strain isolated by Dr. ELLIOTT from maize in Lucas County, Ohio, in October, 1937.

B–109.—A highly virulent strain isolated by Dr. ELLIOTT from the flea beetle vector, *Chaetocnema pulicaria* Melsh., at Arlington, Virginia, in November, 1938.

B–8.—A weakly virulent strain from New York used by Dr. WELLHAUSEN as the parent strain for his host passage studies (**24**). It differed from all other cultures except B–92 in producing a dry, crustaceous type of growth on agar.

B–96.—A highly virulent isolate obtained by Dr. WELLHAUSEN (**24**) from B–8 after a series of passages in a resistant inbred line of maize designated as OSF.

B–91.—A highly virulent isolate obtained from B–8 by a series of host passages similar to those described for B–96 (**24**).

B–94.—An avirulent single-colony isolate obtained from B–96 after it had been cultivated on nutrient-dextrose agar slants for 18 months.

B–92.—A weakly virulent isolate obtained by Dr. WELLHAUSEN (**24**) from culture B–8 after a series of passages in a susceptible inbred line of maize designated as GB.

These different strains were purified by single-colony isolation at the beginning of the experiments in order to eliminate any variant types that might have developed in culture. The isolates were stored on nutrient-dextrose agar at 8° C., and subcultures from this stock were used in all subsequent tests. The cultures were tested for virulence as described elsewhere (**16**) immediately after purification and again at the conclusion of the experiments. No decided changes were observed, so only data on the latter test are reported (table 1).

Immune sera for these strains were obtained from rabbits weighing between 2 and 3.6 kilos. All rabbits were bled at the beginning of the experiment and found to be free of agglutinins for the different bacterial strains. Bacteria grown on potato-dextrose agar slants and suspended in sterile saline solution (0.85 per cent NaCl) were injected intraperitoneally at 4-day intervals. After injections of 1, 1, 3, and 4 cc. of suspension, an injection was made intravenously but the rabbits suffered severe shock and four of the fifteen died. The sixth and final injection

was made intraperitoneally and the rabbits were bled from an ear vein 12 days later. The rabbits lost 0.2–0.7 kilos in weight during the period of injection; some continued to lose until the time of bleeding, and general recovery in weight did not occur until 12 days later. The blood was placed in an icebox overnight, the serum removed by pipette and frozen until it was used for tests.

The serum was diluted 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, and 1:5120 with sterile saline and inoculated with sufficient bacteria to give an opalescent suspension. The bacteria were grown for 24 hours on nutrient-dextrose

TABLE 1

VIRULENCE OF P. STEWARTI STRAINS FOR 7-DAY-OLD SWEET-CORN SEEDLINGS

STRAIN TESTED	PERCENTAGE OF LEAVES INVADED IN DIFFERENT TESTS						DRY WEIGHT PER PLANT (GM.)
	1	2	3	4	5	AVERAGE	
B-11	75.2	67.8	77.4	75.5	77.1	74.6± 3.9	0.31±0.05
B-1011a	0.0	0.0	0.0	0.0	0.0	0.0± 0.0	0.46±0.03
B-1311	70.1	67.3	59.8	58.0	54.9	62.0± 6.4	0.33±0.05
B-1611	0.0	0.0	0.0	0.0	0.0	0.0± 0.0	0.46±0.03
B-1111	0.0	2.6	0.0	1.8	0.0	0.9± 1.2	0.43±0.05
B-102	90.4	66.7	79.8	76.9	79.4	78.6± 8.5	0.32±0.05
B-103	67.9	77.4	83.0	69.0	76.4	74.7± 6.3	0.33±0.05
B-107	87.4	85.3	95.1	89.9	87.5	89.0± 3.8	0.28±0.05
B-108	84.3	90.5	93.1	82.9	92.0	88.6± 4.6	0.22±0.03
B-109	60.4	76.3	67.3	73.1	69.0	69.2± 6.0	0.32±0.05
B-8	34.0	34.8	32.5	36.2	28.2	33.1± 3.1	0.40±0.01
B-96	56.6	82.2	65.4	54.9	77.9	67.4±12.4	0.32±0.05
B-91	76.5	76.9	71.6	88.9	80.0	78.8± 6.4	0.30±0.09
B-94	0.0	0.0	0.0	0.0	0.0	0.0± 0.0	0.49±0.09
B-92	36.2	33.1	28.0	42.3	33.9	34.7± 5.2	0.44±0.06
Uninoculated controls	0.0	0.0	0.0	0.0	0.0	0.0± 0.0	0.51±0.06

agar, suspended in saline and allowed to stand overnight at 8° C., so that all particles of agar and bacterial clumps might settle out. They were then thoroughly mixed with the serum, incubated at 40° C. for 2 hours, and stored overnight at 8° C. before final observations on agglutination were made. The degree of agglutination was recorded as ++++ for complete clearing, +++ for pronounced agglutination without complete clearing, ++ for definite agglutination, + for slight agglutination, ± for doubtful agglutination, and − for no agglutination. The doubtful agglutinations consisted of clouding of the serum without the appearance of macroscopically visible clumps. Clumps of 3–30 cells could be detected by microscopic examination, but only macroscopic tests were made as a general rule.

Experimental results

Agglutination of field isolates in immune serum

In order to determine whether the strains prevalent in maize fields at Princeton, New Jersey, differed in their serological characteristics, ninety single-colony isolates were tested for agglutination in the immune sera of the highly virulent B–96 and slightly virulent B–1111. The field isolates were obtained by methods described elsewhere (**16**) from leaf lesions on naturally infected plants of the various inbred lines listed in table 2. These inbred lines ranged in susceptibility from the completely susceptible C–18 to the highly resistant OSF in the order in which they are listed. Isolates were usually obtained from two or more plants of each inbred line, and two to six isolates from each plant were tested. All the isolates belonged, in so far as could be determined, to the B type described by Ivanoff *et al.* (7). All were highly virulent immediately after isolation, but at the time of the tests, 4 months later, some had partially lost virulence for Golden Bantam seedlings.

The ninety isolates differed widely in their ability to agglutinate in the immune sera. In order to save space, data on only thirty of these isolates are presented. The data in table 2 serve to illustrate the general differences observed. Many of the isolates, such as 3, 4, 6, 15, 25, 26, 28, and 30, agglutinated well in both sera even at dilutions of 1:1280 or more; it is obvious that both sera were fairly potent. It should be pointed out, however, that the B–96 serum caused more complete agglutination at the 1:20 and 1:40 dilutions than did the B–1111 serum. Some of the isolates (2, 11, 19, 21, 22, 23, 29) agglutinated better in B–96 than in B–1111 serum. On the other hand, isolates such as 1, 7, 8, 12, 16, and 27 agglutinated readily in diluted B–1111 serum but poorly in B–96 serum. Isolates such as 9, 10, 13, 17, 18, and 24 agglutinated poorly in both sera, and isolates 14 and 20 failed to agglutinate in either.

The bacteria from resistant and susceptible inbred lines gave similar agglutination reactions, and no consistent difference could be observed in the tendency for atypical strains to occur in the two types of hosts. As a general rule, isolates from the same plant had similar serological characteristics, but in some instances isolates such as 14 and 15 from the same lesion were entirely different. Frequently the isolates from different plants of the same inbred line were just as different from one another as they were from isolates obtained from some other line.

The data on these field isolates show that the agglutination test cannot be used unconditionally as a means of identifying *P. stewarti* cultures. Obviously more than one serum should be tested, but even then the failure of a strain to agglutinate would not disprove its identity. Further tests on morphology, physiology, and virulence would have to be resorted to in identifying these non-agglutinating strains.

TABLE 2

AGGLUTINATION OF BACTERIA ISOLATED FROM NATURALLY INFECTED INBRED LINES OF MAIZE AT PRINCETON, IN 1938

Isolate tested				Agglutination of bacteria in immune serum															
		Virulence		B-96 serum diluted*								B-1111 serum diluted							
Experiment no.	From inbred line	Percentage leaves																	
		Invaded	Killed	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560
1	C-18a†	87.7	51.2	++	±	−	−	−	−	−	−	+	+	+	+	+	+	+	+
2	C-18a†	20.0	1.1	++++	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−
3	C-18b	96.1	55.8	+++	+++	++	++	+	±	+	−	++	+	+	+	+	+	−	−
4	C-18b	87.5	37.5	+++	++	+	+	+	+	+	−	++	++	+	+	+	+	−	−
5	6-565a	84.4	53.3	++++	+	±	−	−	−	−	−	+	+	±	+	+	+	+	±
6	6-565b	91.4	64.3	++	++	+	+	+	+	+	+	+	+	+	+	+	+	±	−
7	GBa	96.2	62.8	++	++	−	−	−	−	−	−	+	+	+	+	+	+	±	−
8	7-40a	80.7	49.4	++++	+++	+	−	−	−	−	−	+	+	+	±	+	+	±	−
9	7-40b	98.4	77.8	+	±	−	−	−	−	−	−	+	±	−	−	−	−	−	−
10	7-40b	98.5	82.4	+	±	−	−	−	−	−	−	±	±	−	−	−	−	−	−
11	7-366a	90.7	53.3	+++	++	+	+	+	±	−	−	++	++	−	−	−	−	±	−
12	6-573a	92.3	65.4	++	+	±	−	−	−	−	−	+	+	+	+	+	+	±	−
13	6-573b	93.0	60.6	+++	±	−	−	−	−	−	−	+	+	±	−	−	−	−	−
14	6-214a	85.3	56.0	±	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
15	6-214a	66.7	32.3	+++	+++	++	+	+	+	±	−	+	+	+	+	+	+	−	−
16	6-214b	83.3	35.7	+++	±	−	−	−	−	−	−	+	+	+	+	+	+	−	−
17	6-195a	89.2	60.2	++	−	−	−	−	−	−	−	+	+	−	−	−	−	−	−
18	6-195a	95.3	76.5	++	−	−	−	−	−	−	−	+	+	−	±	−	−	−	−
19	6-195b	77.4	58.3	+++	+++	+	±	−	−	−	−	−	−	−	−	−	−	−	−
20	Y-30a	82.7	38.7	−	−	−	−	−	−	−	−	±	−	−	−	−	−	−	−
21	6-1494a	76.6	41.6	++	++	±	−	−	−	−	−	−	−	−	−	−	−	−	−
22	6-1494b	87.7	56.8	+++	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−
23	6-1494b	72.6	43.2	+++	++	−	−	−	−	−	−	−	−	−	−	−	−	−	−
24	6-215a	93.8	63.8	+	+	±	−	−	−	−	−	−	±	±	−	−	−	−	−
25	6-215b	85.1	44.8	+++	+++	++	+	+	+	+	±	++	++	+	+	+	+	+	+
26	6-215b	69.8	30.2	+++	++	+	+	+	+	+	+	+	+	+	+	+	+	±	+
27	7-1992a	88.9	52.8	+++	+	±	−	−	−	−	−	+	+	+	+	+	+	+	±
28	7-1992b	86.1	56.9	+++	+++	+	+	+	±	+	−	+	+	+	+	+	+	+	±
29	7-1992b	77.9	42.9	++	+	+	−	−	−	−	−	±	−	−	−	−	−	−	−
30	OSFa	92.3	62.8	+++	+	+	+	+	+	+	±	+	+	+	+	+	+	+	+

* Data on 1:5120 dilution and saline controls omitted, since all tests were negative.

† Small letters indicate different plants of each inbred line; for example, this is plant "a" of inbred line C-18.

Reciprocal agglutination tests on fifteen strains differing in virulence and cultural characteristics

The discovery that serologically distinct variants could be isolated from the same leaf lesion raised the question of whether the plant had been infected originally by a single strain which later produced serological variants or was infected originally by more than one strain. Such a question cannot be answered until it is known whether all the progeny of a given serological type have the same reaction in immune serum. The cross agglutinations of several variant strains obtained from cultures B–11 and B–8 were tested in order to gain information on this point.

TABLE 3

Maximum dilution of eleven immune sera in which fifteen strains of P. stewarti agglutinated*

Strain tested	Maximum dilution at which agglutination occurred in antiserum of strain										
	B-11	B-1011a	B-1611	B-1111	B-102	B-107	B-108	B-8	B-96	B-91	B-94
B-11	1:160	1:40	—†	—	—	—	1:40	—	1:40	1:40	1:40
B-1011a	1:640	1:320	(1:40)‡	1:40	1:320	1:80	1:80	1:40	1:320	1:1280	1:640
B-1311	1:160	1:40	—	—	—	—	1:40	—	1:80	1:40	1:40
B-1611	1:160	1:640	1:1280	1:2560	1:320	1:640	1:320	1:2560	1:160	1:640	1:640
B-1111	1:1280	1:640	1:320	1:2560	1:1280	1:320	1:640	1:2560	1:640	1:1280	1:2560
B-102	1:2560	1:2560	1:1280	1:2560	1:5120	1:2560	1:1280	1:2560	1:5120	1:2560	1:5120
B-103	1:2560	1:40	1:20	1:80	1:120	(1:80)	1:80	1:320	1:40	1:40	1:160
B-107	1:5120	1:160	1:160	1:640	1:320	1:320	1:640	1:320	1:160	1:160	1:2560
B-108	1:320	1:40	1:40	1:40	1:80	1:160	1:80	1:80	1:80	1:320	1:1280
B-109	1:160	1:40	1:80	1:80	1:20	(1:20)	1:320	1:80	1:160	1:40	1:160
B-8	1:2560	1:1280	1:1280	1:2560	1:2560	1:2560	1:1280	1:5120	1:2560	1:2560	1:1280
B-96	1:320	1:320	(1:320)	(1:40)	(1:80)	(1:80)	1:160	(1:160)	1:160	1:320	1:80
B-91	1:160	1:80	(1:80)	—	(1:40)	(1:80)	1:40	—	1:40	1:80	1:40
B-94	1:160	1:160	(1:80)	—	—	—	1:40	—	1:40	1:40	1:40
B-92	1:1280	1:640	1:1280	1:1280	1:640	1:1280	1:320	1:1280	1:160	1:640	1:160

* None of the strains agglutinated when incubated in saline solution.

† No agglutination occurred in either test or at most a doubtful reaction at 1:20 dilution.

‡ Dilution in parentheses represents very faint reaction at this dilution in one test and negative reaction in the other.

Their reactions were also compared with those of several other strains obtained from different geographical areas. The original plan was to compare all fifteen cultures listed in the section on materials, but, as already mentioned, four of the rabbits succumbed and only eleven sera were obtained.

Agglutinations of all fifteen strains in the eleven different sera were tested. Since it was not feasible to test all 165 possible combinations at once, the tests were run on two different dates. In the preliminary tests, half the cultures were tested at one time and the remainder 2 days later. On the repetition, five of the sera were tested against all cultures on the first date and the six remaining sera tested later. Since very similar results were secured in the repetitions, the data are presented without reference to time of test in tables 3, 4, and 5.

* Data on 1:5120 dilution and saline controls omitted, since all tests were negative.
† Small letters indicate different plants of each inbred line; for example, this is plant "a" of inbred line C-18.

The data on the agglutination of all strains in each serum are too voluminous to be presented in a single table, but a summary of the maximum dilution at which

TABLE 4

AGGLUTINATION OF FIFTEEN STRAINS OF P. STEWARTI IN ANTISERUM FOR STRAIN B-11*

STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-11	++++ ++++	++++ ++++	++ ++++	± +	− ±	− −	− −	− −	− −
B-1011a	++++ ++++	++++ ++++	++ ++++	++ +	± +	− +	− +	− −	− −
B-1311	++++ ++++	++++ ++++	++ ++++	+ +	− −	− −	− −	− −	− −
B-1611	++++ +++	++++ +++	+ +++	± ±	− −	− −	− −	− −	− −
B-1111	++++ +++	++++ +++	+++ ++	+++ +	++ +	++ +	++ −	+ −	+ −
B-102	++++ ++++	++++ ++++	++++ ++++	++++ +++	+++ ++	++ +	+ +	+ +	− −
B-103	++++ ++++	++++ ++++	++ ++++	± +	+ ±	+ ±	± ±	± ±	− +
B-107	++++ ++++	++++ ++++	++++ +++	++ +++	++ ++	++ +	++ +	+ +	− +
B-108	++++ ++++	++++ ++++	+++ ++++	+ +	− +	− +	− −	− −	− −
B-109	++++ ++++	++++ ++++	++++ ++++	+ +	± −	− −	− −	− −	− −
B-8	++ ++	+++ +++	+++ +++	+++ ++	+++ +	+++ +	++ +	± +	+ −
B-96	++++ ++++	++++ ++++	++++ +++	+ +	− +	− ±	− −	− −	− −
B-91	++++ +++	++++ ++++	+ ++++	− +	− −	− −	− −	− −	− −
B-94	++++ +++	++++ ++++	+ ++++	− +	− −	− −	− −	− −	− −
B-92	++++ +++	++++ ++++	+ ++++	+ +	+ +	+ +	− +	− −	− −

* None of the strains agglutinated when incubated in saline solution.

agglutination occurred in each test is presented in table 3. In the few instances in which the repetitions were not in exact agreement, a value intermediate between those resulting from the two observations was recorded. The data show that the

strains differed considerably in their agglutination in the different sera. Strains B–8, B–102, B–1111, B–1611, and B–107 agglutinated in all sera and usually at

TABLE 5

AGGLUTINATION OF FIFTEEN STRAINS OF P. STEWARTI IN ANTISERUM FOR STRAIN B-8*

STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
	1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-11	−	−	−	−	−	−	−	−	−
	±	−	−	−	−	−	−	−	−
B-1011a	+	±	−	+	−	−	−	−	−
	±	±	±	−	−	−	−	−	−
B-1311	−	−	−	−	−	−	−	−	−
	−	−	−	−	−	−	−	−	−
B-1611	++	+++	++	++	+	+	+	±	−
	+	+	+	+	+	+	+	+	+
B-1111	++	++	++	++	+	+	+	±	−
	++	++	+	+	+	+	+	+	−
B-102	+++	+++	++	++	++	+	+	±	−
	++	++	+	+	+	+	+	+	−
B-103	++	++	++	+	+	±	−	−	−
	+	+	±	±	±	−	−	−	−
B-107	+++	++	+	±	±	−	−	−	−
	++	++	+	+	±	±	−	−	−
B-108	+	+	±	−	−	−	−	−	−
	+	+	±	±	−	−	−	−	−
B-109	+	++	+	±	±	−	−	−	−
	+	+	±	−	−	−	−	−	−
B-8	+++	+++	+++	+++	+++	++	++	++	+
	++	++	++	++	++	+	+	+	±
B-96	−	−	−	−	−	−	−	−	−
	±	+	+	+	±	±	−	−	−
B-91	−	−	−	−	−	−	−	−	−
	±	−	−	−	−	−	−	−	−
B-94	−	−	−	−	−	−	−	−	−
	±	−	−	−	−	−	−	−	−
B-92	−	+	+	+	+	+	+	+	−
	+	+	+	±	+	+	±	−	−

* None of the strains agglutinated when incubated in saline solution.

the high titers of 1:1280 to 1:5120. Other strains, such as B–11, B–1311, B–96, B–91, and B–94, failed to agglutinate in some of the sera and agglutinated poor-

ly in others. The remaining strains agglutinated in all sera but usually only at the lower dilutions.

The data in table 3 show that closely related strains do not necessarily have the same serological properties. Cultures B–11, B–1011a, B–1311, B–1611, and B–1111 were all single-colony isolates from the same New Jersey culture, yet B–1611 and B–1111 agglutinated much more easily than the other three. Strain B–8 agglutinated much more readily in dilute immune serum than did B–96, B–91, B–94, and B–92, which had been derived from it. Strains derived from the same culture did not agglutinate better in antiserum for each other than did strains obtained from different sources. Some cultures, such as B–11 and B–108, did not agglutinate even in their own sera at high dilutions. Since the failure of these strains to agglutinate in dilute serum cannot be due to lack of specificity, it is probably attributable to their ability to resist agglutination.

The data in table 3 show that some sera were more effective than others. As a general rule the easily agglutinating strains (B–8, B–102, B–107, B–1611, and B–1111) produced serum that agglutinated similar strains but failed to agglutinate B–11, B–1311, B–96, and B–94. These poorly agglutinating strains, on the other hand, produced immune serum that agglutinated all fifteen strains.

Some of the differences observed between the two types of sera are not obvious from the general summary in table 3, so detailed data on the serum for strains B–11 and B–8 are presented in tables 4 and 5. The B–11 serum caused a heavy agglutination of all strains but lost potency for some strains when diluted more than 1:80. Strains such as B–8, B–1111, B–107, and B–102 were agglutinated at higher dilutions. The immune serum for B–8 (table 5) caused its homologous culture to agglutinate well at all dilutions up to 1:5120. The other easily agglutinating strains were similarly affected, but in no case was agglutination at the 1:20, 1:40, and 1:80 dilutions as heavy as that observed in B–11 serum. Several strains failed to agglutinate even at the 1:20 dilution of this serum.

Agglutination of other species of wilt bacteria in immune serum for P. stewarti

A number of wilt-producing bacteria are known to be capable of invading sweet corn and inducing mild symptoms (**25, 16**). Several species of these organisms were tested for agglutination in the immune sera for B–108, B–8, and B–94 to determine whether the reaction was specific for *Phytomonas stewarti*. The species used were *P. insidiosa* (McC.) Bergey *et al.*, *P. michiganensis* (E.F.S.) Bergey *et al.*, *P. solanaceara* (E.F.S.) Bergey *et al.*, *P. sepedonicum* (Spieck.) Bergey *et al.*, *P. flaccumfaciens* (Hedges) Bergey *et al.*, and the motile bacterium isolated from nitrogen-starved maize seedlings by McNew and Spencer (**16**). With the exception of *P. flaccumfaciens*, two isolates of each species were obtained from different sources and tested.

TABLE 6

AGGLUTINATION OF P. STEWARTI AND OTHER SPECIES OF WILT-PRODUCING BACTERIA IN ANTISERUM FOR STRAINS B-108, B-8, AND B-94*

BACTERIA TESTED	ANTI-SERUM OF STRAIN	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
		1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
P. stewarti (8)	B-108	++++	++	±	±	±	±	±	−	−
P. stewarti (8)	B-8	+	±	±	±	+	±	+	+	−
P. stewarti (8)	B-94	−	±	±	±	±	±	±	−	−
P. stewarti (167)	B-108	++++	++++	++	+	+	±	±	−	−
P. stewarti (167)	B-8	++	+	+	+	+	+	+	−	−
P. stewarti (167)	B-94	±	+	+	+	+	±	−	−	−
P. insidiosa (B-33)	B-108	−	−	−	−	−	−	−	+	−
P. insidiosa (B-33)	B-8	−	−	−	−	−	−	−	−	−
P. insidiosa (B-33)	B-94	−	−	−	−	−	−	−	−	−
P. insidiosa (B-34)	B-108	−	−	−	−	−	−	−	−	−
P. insidiosa (B-34)	B-8	−	−	−	−	−	−	−	−	−
P. insidiosa (B-34)	B-94	−	−	−	−	−	−	−	−	−
P. michiganensis (B-51A)	B-108	−	−	−	−	−	−	−	−	−
P. michiganensis (B-51A)	B-8	−	−	−	−	−	−	−	−	−
P. michiganensis (B-51A)	B-94	−	−	−	−	−	−	−	−	−
P. michiganensis (631)	B-108	−	−	−	−	−	−	−	−	−
P. michiganensis (631)	B-8	−	−	−	−	−	−	−	−	−
P. michiganensis (631)	B-94	−	−	−	−	−	−	−	−	−
P. solanaceara (Tom)	B-108	−	−	−	−	−	−	−	−	−
P. solanaceara (Tom)	B-8	−	−	−	−	−	−	−	−	−
P. solanaceara (Tom)	B-94	−	−	−	−	−	−	−	−	−
P. solanaceara (Tob)	B-108	−	−	−	−	−	−	−	−	−
P. solanaceara (Tob)	B-8	−	−	−	−	−	−	−	−	−
P. solanaceara (Tob)	B-94	−	−	−	−	−	−	−	−	−
P. sepedonicum (105)	B-108	−	−	−	−	−	−	−	−	−
P. sepedonicum (105)	B-8	−	−	−	−	−	−	−	−	−
P. sepedonicum (105)	B-94	−	−	−	−	−	−	−	−	−
P. sepedonicum (106)	B-108	−	−	−	−	−	−	−	−	−
P. sepedonicum (106)	B-8	−	−	−	−	−	−	−	−	−
P. sepedonicum (106)	B-94	−	−	−	−	−	−	−	−	−
P. flaccumfaciens (B-43A)	B-108	−	−	−	−	−	−	−	−	−
P. flaccumfaciens (B-43A)	B-8	−	−	−	−	−	−	−	−	−
P. flaccumfaciens (B-43A)	B-94	−	−	−	−	−	−	−	−	−
Motile bacterium (470)	B-108	−	−	−	−	−	−	−	−	−
Motile bacterium (470)	B-8	−	−	−	−	−	−	−	−	−
Motile bacterium (470)	B-94	−	−	−	−	−	−	−	−	−
Motile bacterium (480)	B-108	−	−	−	−	−	−	−	−	−
Motile bacterium (480)	B-8	−	−	−	−	−	−	−	−	−
Motile bacterium (480)	B-94	−	−	−	−	−	−	−	−	−
P. stewarti (B-110)	B-108	++	++	++++	++	++	+	±	−	−
P. stewarti (B-110)	B-8	+	±	+	+	+	±	−	−	−
P. stewarti (B-110)	B-94	++	+	±	−	−	−	−	−	−
P. stewarti (B-111)	B-108	?	?	?	?	?	−	−	−	−
P. stewarti (B-111)	B-8	?	?	?	?	?	−	−	−	−
P. stewarti (B-111)	B-94	?	?	?	−	−	−	−	−	−
P. stewarti (B-112)	B-108	+	+	++	+	−	−	−	−	−
P. stewarti (B-112)	B-8	+	+	±	−	−	−	−	−	−
P. stewarti (B-112)	B-94	±	+	+	±	−	−	−	−	−
P. stewarti (B-113)	B-108	+	+	++	+	+	±	−	−	−
P. stewarti (B-113)	B-8	++	+	+	+	++	+	−	−	−
P. stewarti (B-113)	B-94	+	++	++	++	++	+	−	−	−
P. stewarti (B-114)	B-108	+	±	±	−	−	−	−	−	−
P. stewarti (B-114)	B-8	±	−	−	−	−	−	−	−	−
P. stewarti (B-114)	B-94	−	±	−	−	−	−	−	−	−
P. stewarti (B-115)	B-108	+	+	+	±	±	−	−	−	−
P. stewarti (B-115)	B-8	++	+	+	+	+	±	−	−	−
P. stewarti (B-115)	B-94	+	+	+	+	+	−	−	−	−
P. stewarti (B-116)	B-108	+	++	++	±	−	−	−	−	−
P. stewarti (B-116)	B-8	+	+	+	±	−	−	−	−	−
P. stewarti (B-116)	B-94	+	+	+	+	−	−	−	−	−
P. stewarti (B-117)	B-108	+	++	+	+	+	+	±	−	−
P. stewarti (B-117)	B-8	+++	+++	++	++	+	±	−	−	−
P. stewarti (B-117)	B-94	+	+	+	+	+	+	−	−	−
P. stewarti (B-118)	B-108	++++	++++	++	+	+	±	−	−	−
P. stewarti (B-118)	B-8	+	+	+	+	+	+	±	±	−
P. stewarti (B-118)	B-94	+	+	+	+	+	+	+	+	−
P. stewarti (B-119)	B-108	+	±	±	−	−	−	−	−	−
P. stewarti (B-119)	B-8	−	−	−	−	−	−	−	−	−
P. stewarti (B-119)	B-94	−	−	−	−	−	−	−	−	−

* None of the strains agglutinated when incubated in saline solution.

As a control on the potency of the three sera, two field isolates of *Phytomonas stewarti* and ten strains of *P. stewarti*—obtained from Dr. LINCOLN and numbered B–110 to B–119—were used. Strain B–110 was isolated at Hillsboro, Kentucky, in 1938. All the other strains were derived from a subculture of B–8, but they differed in virulence, colony characteristics, color, and viscosity. B–114, a moderately virulent white type, was not so strongly agglutinated as the other strains. Strain B–111, a mucoid type, formed strings of slime in the immune sera which prevented accurate observation on the extent of agglutination. As may be seen from table 6, the strains of *P. stewarti* were the only cultures to be agglutinated by the immune sera. The failure of the motile bacterium to agglutinate in immune serum brings additional evidence that it is not related to *P. stewarti*, even though it is capable of causing some wilt symptoms. It may be concluded that the agglutination test is probably specific for *P. stewarti*.

Discussion

The agglutination reaction has been advocated by several plant pathologists (**4, 10, 22**) as an aid in identifying bacterial plant pathogens. ST. JOHN-BROOKS *et al.* (**17**) found that phytopathogenic bacteria could be grouped according to their agglutination reactions. GOLDSWORTHY (**4, 5**) used the test in identifying field isolates, while LINK and associates (**9, 10, 11, 18**) made use of reciprocal agglutination tests in determining the group relations of bacteria. Serological methods were widely accepted after it had been demonstrated that strains of *Phytomonas malvacearum* (E.F.S.) Bergey *et al.* (**6, 26**), *P. pisi* (Sack.) Bergey *et al.* (**22**), and *P. tabaca* (W. and F.) Bergey *et al.* (**21**) were identical with other strains of their respective species. STAPP (**21**) suggested that the two bacterial parasites of tobacco, *P. tabaca* and *P. angulata* (F. and M.) Bergey *et al.*, were the same because of their identical serological characteristics. This conclusion has since found support in the data presented by BRAUN (**1**) on the serological characteristics and interconversion of strains of the two bacteria.

Because of the growing tendency to rely upon serological methods in identifying bacteria, BURKHOLDER (**2**) has suggested that data on the reliability of the method were needed. The data presented in this paper show some of the limitations of the agglutination test in so far as it applies to *Phytomonas stewarti*. Strains may differ in their ability to agglutinate in immune sera and to incite agglutinin production in rabbits. In conducting agglutination tests for the identification of cultures, it would be necessary to use more than one serum, and even then the failure of a strain to agglutinate would not constitute evidence that it was not a strain of *P. stewarti*. The identity of such non-agglutinating strains would have to be established by some test other than serological behavior. On the other hand, there seems no good reason why agglutination in immune serum for *P. stewarti* should

not be accepted as evidence that the strain belongs in the species. At least the tests conducted with other closely related species failed to show any lack of specificity.

Apparently the serological characteristics of *P. stewarti* change very easily. For example, several of the variant strains isolated from B–11 were entirely different from the parent strain in their serological reactions. This ability of *P. stewarti* to produce new types probably explains the presence of serologically distinct strains in the same leaf lesion (table 2). It has been shown that strains of *P. tumefaciens* (E.F.S. and Town) Bergey *et al.* (**19**), *Erwinia carotovora* (Jones) Hol. (**12, 20**), and *Rhizobium* sp. (**3, 23**) isolated from different sources differ in their serological properties. A certain degree of stability for the soybean nodule bacterium was indicated by WRIGHT'S (**28**) observations that only one of four serological types could be isolated from any particular nodule and that the isolates did not change during incubation on culture media.

The differences in serological behavior of strains of *P. stewarti* may be due either to differences in antigenic composition or to the ease with which the strains agglutinate. If they possess different antigens they will absorb different types of agglutinins, but agglutinin-absorption tests would have to be made in order to detect these differences. Such tests have been made and will be reported later. It is of interest that the differences in serological behavior of the fifteen strains were not closely correlated with differences in virulence, colony characteristics, or physiological abilities. As a general rule the rough forms of bacteria are more easily agglutinated. B–8, which produced a dry crustaceous growth and approached a rough type, agglutinated very well but not appreciably better than some of the typical smooth types, such as B–102.

Summary

1. The agglutination test was used in an attempt to identify ninety single-colony isolates of *Phytomonas stewarti* which were obtained from twenty-seven naturally infected maize plants at Princeton, New Jersey. Three of the isolates failed to agglutinate in either of two sera used, some agglutinated in one or the other but not in both, and the remainder agglutinated in both sera at dilutions of 1:20 to 1:2560. There was no consistent difference in the serological properties of isolates from susceptible and resistant inbred lines of corn. Some isolates from the same leaf lesion were as different from one another as were those from different plants.

2. Strains derived from the same culture were found to differ in their serological characteristics. A study of fifteen strains showed that the serological properties were not correlated with any particular colony characteristic, physiological ability, or degree of virulence. Strains from different localities in the United States and

Mexico were no more different from one another than some of the variants derived from one of the strains. Some strains agglutinated in all sera at titers as high as 1:1280 to 1:5120. These strongly agglutinating strains usually induced immune sera that failed to agglutinate five of the strains. The strains that agglutinated poorly, on the other hand, produced sera that were effective against all the cultures tested.

3. Several other species of wilt-producing bacteria did not agglutinate in antiserum for three strains of *P. stewarti*.

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AGGLUTININ ABSORPTION BY DIFFERENT STRAINS OF PHYTOMONAS STEWARTI

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Introduction

In previous studies (5), strains of the sweet-corn wilt bacterium, *Phytomonas stewarti* (E. F. S.) Bergey *et al.*, were found to differ in their agglutination reactions. Some of the fifteen strains tested agglutinated readily in both homologous and heterologous immune serum, while others agglutinated poorly or not at all. As a general rule the cultures that agglutinated readily were poor antigens and produced an inferior type of immune serum. It was considered probable that the differences in the agglutination reactions resulted not alone from distinct serological differences in the strains but also from the inagglutinability of certain strains. Since the absorption of agglutinins by bacterial strains is generally recognized as a reliable serological method for determining relationships, reciprocal agglutinin-absorption tests were made for several of the agglutinable and inagglutinable strains. The results are presented in this paper.

Material and methods

The sera and cultures used in previous tests (5) were employed. The cultures had been kept on nutrient-dextrose agar slants at 8° C. and the sera had been frozen and stored in an icebox. So far as could be determined, neither the cultures nor the sera had changed appreciably during the 2 months of storage under these conditions.

Since it was not feasible to run absorption tests on all the sera at once, only two or three sera and their homologous bacterial strains were used in each of a series of five tests. As a general rule each test included one of the strains that agglutinated readily, one that agglutinated poorly, and a third that was closely related to one of the other strains in that it was derived from the same culture but differed from it in virulence, cultural characteristics, or physiological abilities. The antisera for the different strains used in each test were thawed and divided into three samples consisting of 1 cc. each. Each sample was absorbed by one or the other of the three strains. As a control, a fourth sample of each serum was diluted in saline and kept by the side of the other three during the absorptions.

The absorptions were conducted according to routine methods. Bacteria were grown on nutrient-dextrose agar plates and suspended in sterile saline. Usually 3 or 4 cc. of concentrated bacterial suspension was introduced into each sample

of serum, thoroughly mixed by shaking, and incubated at 40° C. for 2 hours. The bacteria and their absorbed agglutinins were then centrifuged from the suspension and a fresh lot of bacteria added. After five or six such absorptions, when the sera had lost much of their potency, they were mixed with fresh lots of bacteria and stored overnight at 8° C. to complete the absorption. Such absorbed sera were centrifuged to remove as many bacteria as possible and sufficient saline added to give a final dilution of 1:10 or 1:20.

The absorbed sera and their unabsorbed controls were divided into 1 cc. samples, diluted in saline, and tested for agglutinins by the methods described previously (5). Samples of the unabsorbed sera were tested against the three strains used in that particular test. The absorbed sera were tested against all fifteen strains except where otherwise noted in the text. In tests where the absorbed sera had been cleared of agglutinins, only the data on the three strains used in that particular test are reported.

Experimental results

Strains B-1611, B-1111, and B-91 and their immune sera were used in the first test. B-91 was a highly virulent type that agglutinated poorly, while B-1611 and B-1111 were practically avirulent and agglutinated readily. B-1611 and B-1111 were derived from strain B-11 but differed in their physiological properties. B-1611 used inorganic nitrogen while B-1111 did not. Each of the three cultures absorbed the agglutinins from their own serum as well as from the sera for the other two cultures. The absorbed sera were tested for residual agglutinins. None of them caused agglutination, as may be seen from table 1. Since each strain absorbed the agglutinins produced by the other two, it may be concluded that all three were identical in their antigenic composition. The other twelve strains also tested against the absorbed sera were not agglutinated.

It is of interest that B-91, which failed to agglutinate in immune serum for B-1611 and which gave doubtful agglutination in the immune serum for B-1111, had absorbed all the agglutinins from these two sera. This strain apparently failed to agglutinate because it was resistant to the physical forces that cause agglutination of the bacterial cells. Further evidence of resistance to agglutination is brought out by the fact that B-91 agglutinated poorly in its own serum. Unless it be postulated that some kind of non-specific agglutinin absorption was involved, it must be concluded that strains B-91, B-1111, and B-1611 are of identical antigenicity in spite of the differences noted in table 1 and elsewhere (5) on their ability to agglutinate. The possibility of non-specific absorption seems rather remote in view of the results obtained with other strains in the third and fourth tests.

In the second test, strains B-102, B-96, and B-94 and their immune sera were

used. Strain B-102 agglutinated easily and had produced an immune serum that distinctly failed to agglutinate other strains such as B-94 and B-96. The other two strains agglutinated poorly but had produced fairly potent sera. Both B-96

TABLE 1

REACTIONS OF THREE STRAINS OF P. STEWARTI IN ABSORBED AND UNABSORBED IMMUNE SERA FOR B-1611, B-1111, AND B-91*

ANTI-SERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-1611	B-1611	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	B-1111	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	B-91	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	Unabsorbed	B-1611	+	+	+	+	+	−	−	−	−
	"	B-1111	+	+	+	+	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
B-1111	B-1611	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	B-1111	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	B-91	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	Unabsorbed	B-1611	±	+	+	+	+	+	+	+	−
	"	B-1111	±	+	+	+	+	−	−	−	−
	"	B-91	±	−	−	−	−	−	−	−	−
B-91	B-1611	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	B-1111	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	B-91	B-1611	−	−	−	−	−	−	−	−	−
	"	B-1111	−	−	−	−	−	−	−	−	−
	"	B-91	−	−	−	−	−	−	−	−	−
	Unabsorbed	B-1611	+	+	+	+	+	−	−	−	−
	"	B-1111	+++	++	+	+	+	+	−	−	−
	"	B-91	++	+	−	−	−	−	−	−	−

* None of the strains agglutinated when incubated in saline control.

and B-94 were obtained from strain B-8, but B-96 was highly virulent and B-94 avirulent. After the immune sera for these three strains had been absorbed five times by the homologous and heterologous cultures, they were tested against all fifteen strains for residual agglutinins. The data on these tests are not presented,

since each culture had absorbed all the agglutinins from the three sera. Apparently these strains were of identical antigenic composition.

Strains B-8 and B-11 were selected for the third test. These two cultures were characterized by the ease with which B-8 agglutinated at high titers in all immune sera used and by the poor agglutinability of B-11. The immune serum produced by B-11 was the most generally effective of those tested (5), while that of B-8 failed to agglutinate five of the fifteen strains, including B-11.

These two cultures proved to be distinctly different in their abilities to incite and absorb agglutinins. As shown in table 2, B-8 cleared the B-11 immune serum of agglutinins effective against itself without removing the agglutinins effective against B-11 and the other strains. In the reciprocal absorption, B-11 failed to remove all the agglutinins from the B-8 immune serum. The residual agglutinins were effective against B-8 and B-107. This suggests that a closer relationship existed between the New York (B-8) and Mexico (B-107) strains than between either of these and the New Jersey (B-11) strain. In addition to the specific agglutinins produced by each strain and not absorbed by the other, apparently each culture produced some agglutinins that were absorbed by the other. At least the cross-absorptions reduced the titer and intensity of agglutination in the two sera. This suggests that the two strains are similar in some respects even though they may be distinguished by certain specific differences.

The residual agglutinins in the absorbed sera do not appear to be the result of incomplete absorptions, because both cultures had cleared the heterologous sera of agglutinins effective against themselves. Furthermore, both had cleared their own homologous sera of agglutinins by an identical treatment. Of course, the failure of B-11 to agglutinate in absorbed B-8 serum cannot be accepted as evidence that all agglutinins had been removed, since this culture would not agglutinate even in unabsorbed serum. The failure of B-8 to show definite agglutination in absorbed B-11 serum gave conclusive evidence that agglutinins for this culture had been removed.

Strains B-1011a, B-107, and B-108 were used in the fourth test. The three strains were obtained from different geographical areas. B-1011a was avirulent while the other two were highly virulent. Reciprocal absorptions were made on the antiserum of each. After five absorptions, the sera were tested for residual agglutinins against all fifteen strains. The data obtained on the sera absorbed by different strains are presented in table 3. Where the sera had been cleared of agglutinins, only data on B-1011a, B-107, and B-108 are presented. The data on all fifteen strains are given only for those sera that had residual agglutinins.

Strains B-1011a and B-108 appeared identical in their antigenic composition, since each completely absorbed the agglutinins produced by the other. B-107 differed from the other two in ability to absorb agglutinins. It failed to clear B-108

TABLE 2

AGGLUTINATION OF P. STEWARTI CULTURES IN ABSORBED AND UNABSORBED IMMUNE SERA FOR CULTURES B-11 AND B-8*

ANTI-SERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-11	B-8	B-11	+	++	++	+	−	−	−	−	−
	B-8	B-1311	++	++	+	−	−	−	−	−	−
	B-8	B-1011a	++	++	+	+	−	−	−	−	−
	B-8	B-1611	++	++	++	−	−	−	−	−	−
	B-8	B-1111	+++	+	−	−	−	−	−	−	−
	B-8	B-107	+	+	+	+	−	−	−	−	−
	B-8	B-108	++	+	+	+	−	−	−	−	−
	B-8	B-109	+	+	+	−	−	−	−	−	−
	B-8	B-102	++	+	+	+	±	−	−	−	−
	B-8	B-103	++	+	+	−	−	−	−	−	−
	B-8	B-8	±	−	−	−	−	−	−	−	−
	B-8	B-96	+++	++	+	−	−	−	−	−	−
	B-8	B-92	+	+	+	+	−	−	−	−	−
	B-8	B-91	+	+	+	+	−	−	−	−	−
	B-8	B-94	+++	+	+	+	−	−	−	−	−
B-11	B-11	B-11	−	−	−	−	−	−	−	−	−
	B-11	B-1311	−	−	−	−	−	−	−	−	−
	B-11	B-1011a	−	−	−	−	−	−	−	−	−
	B-11	B-1611	−	−	−	−	−	−	−	−	−
	B-11	B-1111	−	−	−	−	−	−	−	−	−
	B-11	B-107	−	−	−	−	−	−	−	−	−
	B-11	B-108	−	−	−	−	−	−	−	−	−
	B-11	B-109	−	−	−	−	−	−	−	−	−
	B-11	B-102	−	−	−	−	−	−	−	−	−
	B-11	B-103	−	−	−	−	−	−	−	−	−
	B-11	B-8	−	−	−	−	−	−	−	−	−
	B-11	B-96	−	−	−	−	−	−	−	−	−
	B-11	B-92	−	−	−	−	−	−	−	−	−
	B-11	B-91	−	−	−	−	−	−	−	−	−
	B-11	B-94	−	−	−	−	−	−	−	−	−
B-11	Unabsorbed	B-11	++++	+++	+++	±	−	−	−	−	−
	Unabsorbed	B-8	++	++	++	+	+	+	±	±	−
B-8	B-11	B-11	−	−	−	−	−	−	−	−	−
	B-11	B-1311	−	−	−	−	−	−	−	−	−
	B-11	B-1011a	−	−	−	−	−	−	−	−	−
	B-11	B-1611	−	−	−	−	−	−	−	−	−
	B-11	B-1111	−	−	−	−	−	−	−	−	−
	B-11	B-107	+	+	±	−	−	−	−	−	−
	B-11	B-108	−	−	−	−	−	−	−	−	−
	B-11	B-109	−	−	−	−	−	−	−	−	−
	B-11	B-102	±	−	−	−	−	−	−	−	−
	B-11	B-103	−	−	−	−	−	−	−	−	−
	B-11	B-8	+	+	+	+	−	−	−	−	−
	B-11	B-96	−	−	−	−	−	−	−	−	−
	B-11	B-92	−	−	−	−	−	−	−	−	−
	B-11	B-91	−	−	−	−	−	−	−	−	−
	B-11	B-94	−	−	−	−	−	−	−	−	−

* None of the strains agglutinated when incubated in saline control.

TABLE 2—*Continued*

Anti-serum for strain	Serum absorbed by strain	Strain tested	Agglutination of bacteria in serum diluted								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-8	B-8	B-11	−	−	−	−	−	−	−	−	−
	B-8	B-1311	−	−	−	−	−	−	−	−	−
	B-8	B-1011a	−	−	−	−	−	−	−	−	−
	B-8	B-1611	−	−	−	−	−	−	−	−	−
	B-8	B-1111	−	−	−	−	−	−	−	−	−
	B-8	B-107	−	−	−	−	−	−	−	−	−
	B-8	B-108	−	−	−	−	−	−	−	−	−
	B-8	B-109	−	−	−	−	−	−	−	−	−
	B-8	B-102	−	−	−	−	−	−	−	−	−
	B-8	B-103	−	−	−	−	−	−	−	−	−
	B-8	B-8	−	−	−	−	−	−	−	−	−
	B-8	B-96	−	−	−	−	−	−	−	−	−
	B-8	B-92	−	−	−	−	−	−	−	−	−
	B-8	B-91	−	−	−	−	−	−	−	−	−
	B-8	B-94	−	−	−	−	−	−	−	−	−
B-8	Unabsorbed	B-11	−	−	−	−	−	−	−	−	−
	Unabsorbed	B-8	++	+	+	+	+	+	+	−	−

antiserum of agglutinins for several other strains. On the other hand, B-1011a failed to clear B-107 antiserum of agglutinins. The fact that the residual agglutinins were effective against both B-107 and B-8 suggests that these two strains have something in common, as mentioned in discussing the data obtained in the third test.

In order to determine whether B-8 and B-107 would give comparable reactions when tested under similar conditions, a fifth experiment was made with these strains and B-11. Six absorptions were made with each strain on separate samples of the three homologous and heterologous sera, and then the sera were used for agglutination tests against strains B-107, B-8, B-11, B-102, B-1111, B-92, and B-94. The data confirm those reported in tables 2 and 3. B-11 failed to clear either B-8 or B-107 immune sera of agglutinins for these two strains. On the other hand, B-8 and B-107 failed to clear the antiserum for B-11 of agglutinins effective against the other strains.

The facts that B-11 failed to remove agglutinins from antisera for either B-107 or B-8, and that the residual agglutinins were effective against both strains, suggest that they are closely related. However, B-107 and B-8 left agglutinins in B-11 serum that were effective against each other. It follows that if the cultures were antigenically distinct, they would not completely absorb the agglutinins from their respective heterologous sera. As shown in table 4, however, they did completely absorb each other's agglutinins. The answer to this discrepancy in the results is not immediately obvious. In repetitions of the experiment it was observed that

TABLE 3

AGGLUTINATION OF P. STEWARTI CULTURES IN ABSORBED AND UNABSORBED IMMUNE SERA FOR B-1011a, B-107, AND B-108*

ANTI-SERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN SERUM DILUTED								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-107	B-107	B-1011a	−	−	−	−	−	−	−	−	−
	B-107	B-107	−	−	−	−	−	−	−	−	−
	B-107	B-108	−	−	−	−	−	−	−	−	−
	B-1011a	B-11	−	−	−	−	−	−	−	−	−
	B-1011a	B-1311	−	−	−	−	−	−	−	−	−
	B-1011a	B-1011a	−	−	−	−	−	−	−	−	−
	B-1011a	B-1611	−	−	−	−	−	−	−	−	−
	B-1011a	B-1111	−	−	−	−	−	−	−	−	−
	B-1011a	B-107	+	+	±	±	−	−	−	−	−
	B-1011a	B-108	−	−	−	−	−	−	−	−	−
	B-1011a	B-109	−	−	−	−	−	−	−	−	−
	B-1011a	B-102	−	−	−	−	−	−	−	−	−
	B-1011a	B-103	−	−	−	−	−	−	−	−	−
	B-1011a	B-8	±	+	+	±	−	−	−	−	−
	B-1011a	B-96	−	−	−	−	−	−	−	−	−
	B-1011a	B-92	−	−	−	−	−	−	−	−	−
	B-1011a	B-91	−	−	−	−	−	−	−	−	−
	B-1011a	B-94	−	−	−	−	−	−	−	−	−
	B-108	B-11	−	−	−	−	−	−	−	−	−
	B-108	B-1311	−	−	−	−	−	−	−	−	−
	B-108	B-1011a	−	−	−	−	−	−	−	−	−
	B-108	B-1611	−	−	−	−	−	−	−	−	−
	B-108	B-1111	−	−	−	−	−	−	−	−	−
	B-108	B-107	−	−	−	−	−	−	−	−	−
	B-108	B-108	−	−	−	−	−	−	−	−	−
	B-108	B-109	−	−	−	−	−	−	−	−	−
	B-108	B-102	−	−	−	−	−	−	−	−	−
	B-108	B-103	−	−	−	−	−	−	−	−	−
	B-108	B-8	+	+	+	+	−	−	−	−	−
	B-108	B-96	−	−	−	−	−	−	−	−	−
	B-108	B-92	−	−	−	−	−	−	−	−	−
	B-108	B-91	−	−	−	−	−	−	−	−	−
	B-108	B-94	−	−	−	−	−	−	−	−	−
	Unabsorbed	B-1011a	−	−	−	−	−	−	−	−	−
	Unabsorbed	B-107	±	+	+	+	+	+	±	−	−
	Unabsorbed	B-108	−	±	±	−	−	−	−	−	−
B-1011a	B-1011a	B-1011a	−	−	−	−	−	−	−	−	−
	B-1011a	B-107	−	−	−	−	−	−	−	−	−
	B-1011a	B-108	−	−	−	−	−	−	−	−	−
	B-107	B-1011a	−	−	−	−	−	−	−	−	−
	B-107	B-107	−	−	−	−	−	−	−	−	−
	B-107	B-108	−	−	−	−	−	−	−	−	−
	B-108	B-1011a	−	−	−	−	−	−	−	−	−
	B-108	B-107	−	−	−	−	−	−	−	−	−
	B-108	B-108	−	−	−	−	−	−	−	−	−
	Unabsorbed	B-1011a	+++	+	−	−	−	−	−	−	−
	Unabsorbed	B-107	+	+	+	±	±	−	−	−	−
	Unabsorbed	B-108	+	+	−	−	+	−	−	−	−

* None of the strains agglutinated when incubated in saline control.

TABLE 3—*Continued*

Anti-serum for strain	Serum absorbed by strain	Strain tested	Agglutination of bacteria in serum diluted								
			1:20	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
B-108	B-108	B-1011a	−	−	−	−	−	−	−	−	−
	B-108	B-107	−	−	−	−	−	−	−	−	−
	B-108	B 108	−	−	−	−	−	−	−	−	−
	B-107	B-11	−	±	−	−	−	−	−	−	−
	B-107	B-1311	−	−	−	−	−	−	−	−	−
	B-107	B-1011a	−	−	−	−	−	−	−	−	−
	B-107	B-1611	−	±	−	−	−	−	−	−	−
	B-107	B-1111	+	+	+	+	−	−	−	−	−
	B-107	B-107	−	−	−	−	−	−	−	−	−
	B-107	B-108	±	+	±	−	−	−	−	−	−
	B-107	B-109	−	−	−	−	−	−	−	−	−
	B-107	B-102	−	−	−	−	−	−	−	−	−
	B-107	B-103	±	±	−	−	−	−	−	−	−
	B-107	B-8	+	+	±	−	−	−	−	−	−
	B-107	B-96	−	+	±	±	−	−	−	−	−
	B-107	B-92	±	+	+	±	±	−	−	−	−
	B-107	B-91	−	±	+	−	−	−	−	−	−
	B-107	B-94	−	−	−	−	−	−	−	−	−
	B-1011a	B-1011a	−	−	−	−	−	−	−	−	−
	B-1011a	B-107	−	−	−	−	−	−	−	−	−
	B-1011a	B-108	−	−	−	−	−	−	−	−	−
	Unabsorbed	B-1011a	+++	++	+	±	−	−	−	−	−
	Unabsorbed	B-108	++	+	+	−	−	−	−	−	−
	Unabsorbed	B-107	++	++	+	+	+	±	−	−	−

some residual agglutinins were present in immune sera for B-107 and B-8 after they had been absorbed by the heterologous cultures. The amount of residual agglutinins was so small that their effects could be detected only by microscopic examination of the bacterial suspension in the absorbed sera. Small clumps of 3–30 cells could be detected, particularly in B-8 immune serum absorbed by B-107 and tested against B-8. Similar clumps were not observed in the immune sera that had been absorbed by their homologous strains. It is believed, therefore, that B-107 and B-8 are not identical, even though they are very similar in most of their antigenic properties. The agglutinins which each failed to absorb from antiserum of the other were a minor fraction of the total.

Discussion

Many strains of *Phytomonas stewarti* have been identified in recent years (**1, 2, 3, 4, 6, 7**) by differences in their virulence, colony characteristics, color, or physiological abilities. According to IVANOFF *et al.* (**1**), at least three major types exist under field conditions. In view of these differences, it is not surprising that some strains should also differ in their antigenic composition. No correlation could be detected,

TABLE 4

AGGLUTINATION OF P. STEWARTI IN ABSORBED IMMUNE SERA OF CULTURES B-11, B-107, AND B-8*

ANTISERUM FOR STRAIN	SERUM ABSORBED BY STRAIN	STRAIN TESTED	AGGLUTINATION OF BACTERIA IN IMMUNE SERUM DILUTED					
			1:10	1:20	1:40	1:80	1:160	1:320
B-11	B-107	B-107	−	−	−	−	−	−
	B-107	B-11	+	+	++	+	+	±
	B-107	B-8	+	+	+	+	+	±
	B-107	B-102	+	+	+	+	+	+
	B-107	B-1111	±	±	±	±	−	−
	B-107	B-92	±	+	−	−	−	−
	B-107	B-94	−	−	−	−	−	−
B-11	B-8	B-107	+++	+	−	−	−	−
	B-8	B-11	+++	+	−	−	−	−
	B-8	B-8	−	−	−	−	−	−
	B-8	B-102	±	+	+	+	−	−
	B-8	B-1111	−	−	−	−	−	−
	B-8	B-92	+	+	+	+	−	−
	B-8	B-94	+	+	+	+	−	−
B-11	B-11	B-107	−	−	−	−	−	−
	B-11	B-11	−	−	−	−	−	−
	B-11	B-8	−	−	−	−	−	−
B-11	Unabsorbed	B-107	.....	+++	+++	+++	++	++
	Unabsorbed	B-11	.....	++++	++++	+++	±	±
	Unabsorbed	B-8	.....	++	++	++	+	+
B-107	B-8	B-107	−	−	−	−	−	−
	B-8	B-11	−	−	−	−	−	−
	B-8	B-8	−	−	−	−	−	−
	B-8	B-102	−	−	−	−	−	−
	B-8	B-1111	−	−	−	−	−	−
	B-8	B-92	−	−	−	−	−	−
	B-8	B-94	−	−	−	−	−	−
B-107	B-11	B-107	+	+	+	+	−	−
	B-11	B-11	−	−	−	−	−	−
	B-11	B-8	+	+	+	±	−	−
	B-11	B-102	−	−	−	−	−	−
	B-11	B-1111	−	−	−	−	−	−
	B-11	B-92	−	−	−	−	−	−
	B-11	B-94	−	−	−	−	−	−
B-107	B-107	B-107	−	−	−	−	−	−
	B-107	B-11	−	−	−	−	−	−
	B-107	B-8	−	−	−	−	−	−
B-107	Unabsorbed	B-107	.....	+	++	++	++	++
	Unabsorbed	B-11	.....	±	−	−	−	−
	Unabsorbed	B-8	.....	+	+	+	+	+

* None of the strains agglutinated when incubated in saline control.

TABLE 4—*Continued*

Antiserum for strain	Serum absorbed by strain	Strain tested	Agglutination of bacteria in immune serum diluted					
			1:10	1:20	1:40	1:80	1:160	1:320
B-8	B-107	B-107	−	−	−	−	−	−
	B-107	B-11	−	−	−	−	−	−
	B-107	B-8	−	−	−	−	−	−
	B-107	B-102	−	−	−	−	−	−
	B-107	B-1111	−	−	−	−	−	−
	B-107	B-92	−	−	−	−	−	−
	B-107	B-94	−	−	−	−	−	−
B-8	B-11	B-107	+	+	+	−	−	−
	B-11	B-11	−	−	−	−	−	−
	B-11	B-8	+	+	+	+	±	−
	B-11	B-102	−	−	−	−	−	−
	B-11	B-1111	−	−	−	−	−	−
	B-11	B-92	−	−	−	−	−	−
	B-11	B-94	−	−	−	−	−	−
B-8	B-8	B-107	−	−	−	−	−	−
	B-8	B-11	−	−	−	−	−	−
	B-8	B-8	−	−	−	−	−	−
B-8	Unabsorbed	B-107	.....	+	+	+	+	±
	Unabsorbed	B-11	.....	−	−	−	−	−
	Unabsorbed	B-8	.....	++	++	+	+	+

however, between antigenic composition and other characteristics. For example, in some species of bacteria rough and smooth types are serologically distinct. In *P. stewarti* the firm crustaceous B-8 was very similar to the soft spreading B-107, and both were distinctly different from the smooth B-11. There was also no correlation between virulence and any particular antigenic type. Avirulent B-94 and highly virulent B-96 and B-102 were found to be closely related, while highly virulent B-11 and B-107 proved to be distinctly different in their serological characteristics.

The differences previously reported (**5**) on the agglutinability of the strains in immune serum can be interpreted in view of the present data. Some of the strains do produce and absorb specific agglutinins; but these specific types are only a fraction of the total and are not the major factor responsible for differences in agglutination. For example, B-94 failed to agglutinate in B-102 immune serum yet it absorbed the agglutinins just as effectively as did B-102, which agglutinated readily. Similarly, B-11 failed to agglutinate in the immune serum of B-8 in spite of the fact that it absorbed all the agglutinins except a minor type specialized for B-8. Conversely, B-8 agglutinated readily in B-11 immune serum even though it absorbed only a fraction of the agglutinins. Such differences in agglutinability might well be attributed to the resistance of the different strains to the physical

factors that cause agglutination after the agglutinins have been absorbed. These differences might have been anticipated because it is known that some strains of other species differ in the ease with which they agglutinate in saline solution. It follows that the simple agglutination test is inadequate in determining differences in the antigenic constituents of bacteria.

Summary

1. Strains of *Phytomonas stewarti* that differed in virulence, colony type, physiology, and agglutination reaction were tested for ability to absorb agglutinins from immune sera. All the strains tested absorbed most of the agglutinins from both homologous and heterologous sera, indicating that they were similar in most essentials. The failure of some strains to agglutinate after absorbing agglutinins was apparently due to their resistance to this reaction.

2. Some differences were observed between the strains from Mexico (B-107), New York (B-8), and New Jersey (B-11). The strains from Mexico and New York failed to absorb all the agglutinins from the antiserum for the New Jersey strain. In reciprocal tests, the New Jersey strain failed to absorb all the agglutinins from immune sera of the other two strains.

3. The differences in serological properties were not closely correlated with any other characteristic, such as virulence or colony type.

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NITROGEN METABOLISM OF THE PLANT EMBRYO

W. L. MCRARY

(WITH FOUR FIGURES)

Introduction

In connection with recent studies on the protein metabolism of grasses and legumes, a new system of microanalysis for protein and its degradation products was developed by BORSOOK and DUBNOFF (2). Using these methods, these workers found that both grasses and legumes synthesized protein in the growing regions during germination, both in the light and darkness. Since such plants are relatively high in protein and carbohydrate reserves, it seemed of interest to investigate a plant belonging to the high-protein low-carbohydrate reserve class (Prianischnikow's third type). The present study deals with the lupin, a typical example of the latter group.

Morphologically the bulk of the lupin seed consists of two cotyledons which contain the reserve food supply. During germination the protein or its decomposition products are translocated downward from the cotyledons into the developing stem and root, and used in forming these new tissues. By analyzing separately the cotyledons and the stem and root (which together will be called the axis) at various stages of development, it is possible to follow the course of the nitrogen metabolism with regard to the site as well as to the nature of the changes involved.

Treatment of the tissue with hot water or alcohol, or drying or freezing, tends to change the original form of the nitrogenous constituents (4). Proteins and amides are especially labile to such treatment, and since these compounds were of particular interest in this study, less drastic treatment was sought. The method finally adopted consisted of repeated extractions and grinding of the fresh material with dilute Na_2SO_4 solution, which dissolves both albumins and globulins as well as the less highly organized nitrogen compounds. By this method "insoluble nitrogen," the nature of which is different in each case according to the technique of extraction used, was reduced to less than 5 per cent of the total nitrogen. Determinations on such a complete extract can then serve better to identify the various forms of nitrogen, as well as to indicate more clearly the relationships between them.

Experimentation

In a series of initial experiments, several nitrogen-free inorganic salt mixtures were tested as culture media. Analysis of the plants after 14 days' germination, however, showed that these media had no detectable effect on the nitrogen metabolism as compared with water controls, hence redistilled water alone was used.

The seeds used were the Hartwegii species of dark blue lupin, *Lupinus hartwegii*, obtained from the Ferry-Morse Seed Company. About one hundred seeds were soaked in redistilled water for 3 hours, then placed individually in vials containing water-saturated cotton. The vials were placed in a large glass-covered box in a constant temperature darkroom (25° C.) free from laboratory fumes. A large open vessel of water in the box prevented excess evaporation of moisture from the cotton. Continuous illumination was provided by a 100-watt lamp 18 inches above the vials. The etiolated plants received the same treatment except for the illumination.

At the end of the period of germination, the embryos were separated from the cotton, the seedcoats removed, and excess moisture taken up with filter paper. The cotyledons were cut off at their junction with the axis, and axis and cotyledons weighed and handled separately in all subsequent treatment.

The tissue from fifty plants was placed in a small heavy-walled test tube fitted to receive a glass grinding rod. A small amount of washed sand was added and the tissue ground to a fine pulp. One ml. of 4 per cent Na_2SO_4 solution was added and the grinding continued another minute. The mixture was centrifuged and the supernatant transferred to a 10-ml. volumetric flask. This process was repeated until the supernatant was clear; the extract was then made to volume and the nitrogen determinations carried out by the method of BORSOOK and DUBNOFF. Water content was determined by the difference in weight of a sample of the tissue before and after drying at 100° C. for 10 hours. In addition to the nitrogen fractions shown in the following section, ammonia nitrogen was determined by micro distillation and peptide nitrogen by the use of a peptidase mixture described by ORCUTT and WILSON (4).

Results

The results are expressed graphically in figures 1–4, in which the various forms of nitrogen (as percentage of total plant nitrogen) are plotted against the time in days. The total nitrogen in one hundred plants averaged 102 mg. The concentrations of amide and amino nitrogen in the water of the tissues are given in tables 1–4.

PLANTS GROWN IN DARKNESS

Figures 1 and 2 show that nitrogen is transferred from the cotyledons to the axis for about the first 12 days. This increase in total axis nitrogen is paralleled closely by a decrease in the protein nitrogen of the cotyledon. The protein nitrogen of the axis and the amino and amide nitrogen of the cotyledon show relatively little change during the period studied.

From the values given in table 1 it is seen that the concentration of amino nitrogen, and hence of amino acids, remains fairly constant in the axis, dropping to a

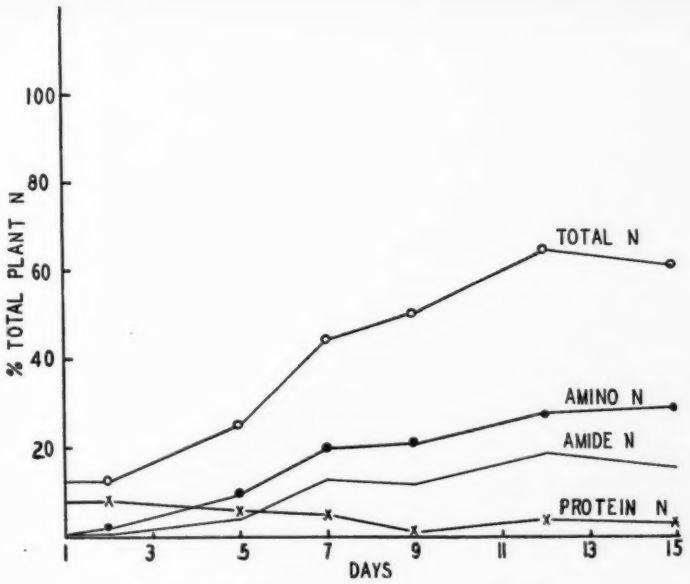

FIG. 1.—Etiolated series: Nitrogen distribution in axis

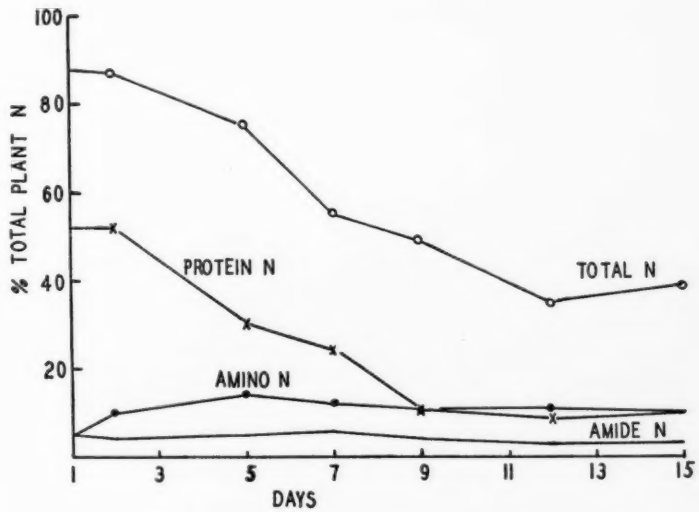

FIG. 2.—Etiolated series: Nitrogen distribution in cotyledons

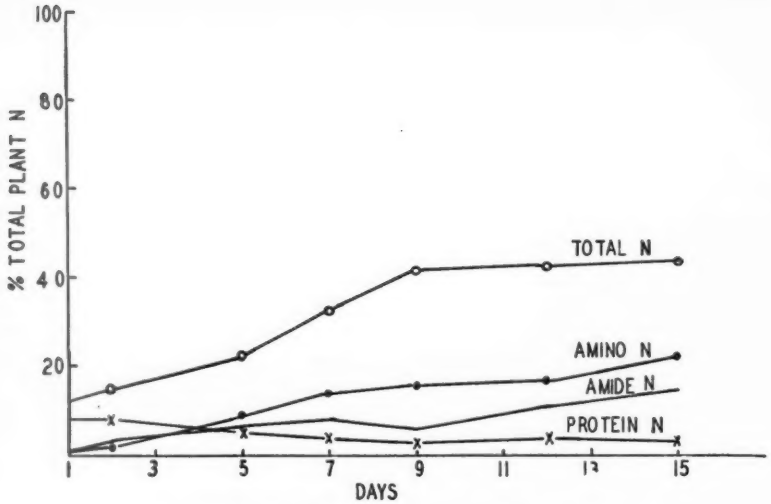

FIG. 3.—Illuminated series: Nitrogen distribution in axis

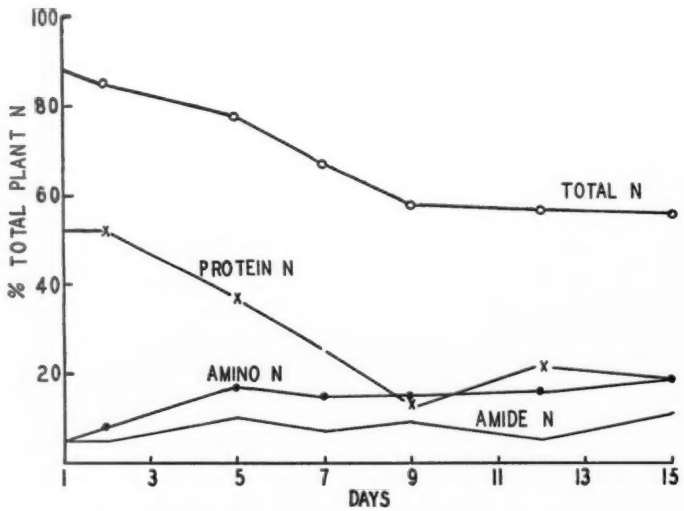

FIG. 4.—Illuminated series: Nitrogen distribution in cotyledons

TABLE 1

MOLAL CONCENTRATION OF NITROGEN FRACTIONS IN WATER OF TISSUE. AXIS; ETIOLATED SERIES

NITROGEN	TIME IN DAYS						
	1	2	5	7	9	12	15
Amino	0.19	0.17	0.23	0.14	0.16	0.16	0.11
Amide	0.09	0.05	0.09	0.09	0.09	0.11	0.06

TABLE 2

MOLAL CONCENTRATION OF NITROGEN FRACTIONS IN WATER OF TISSUE. COTYLEDONS; ETIOLATED SERIES

NITROGEN	TIME IN DAYS						
	1	2	5	7	9	12	15
Amino	0.18	0.34	0.35	0.25	0.27	0.24	0.19
Amide	0.16	0.14	0.13	0.13	0.09	0.08	0.06

TABLE 3

MOLAL CONCENTRATION OF NITROGEN FRACTIONS IN WATER OF TISSUE. AXIS; ILLUMINATED SERIES

NITROGEN	TIME IN DAYS						
	1	2	5	7	9	12	15
Amino	0.19	0.16	0.29	0.14	0.16	0.16	0.16
Amide	0.09	0.21	0.16	0.09	0.06	0.10	0.11

TABLE 4

MOLAL CONCENTRATION OF NITROGEN FRACTIONS IN WATER OF TISSUE. COTYLEDONS; ILLUMINATED SERIES

NITROGEN	TIME IN DAYS						
	1	2	5	7	9	12	15
Amino	0.18	0.23	0.34	0.23	0.17	0.16	0.18
Amide	0.16	0.14	0.19	0.11	0.10	0.05	0.10

somewhat lower value by the fifteenth day. Corresponding values for the amino nitrogen in the cotyledons are at a higher level. With the exception of the irregularity on the second day, the amide concentration in the axis remains constant up to the twelfth day, after which it drops. The amide concentration in the cotyledons decreases steadily throughout the period studied.

Plants grown in light

The data for the axis are shown in figure 3. The increase in total nitrogen is less rapid than in the dark, and reaches a constant value by the ninth day. At this time the plant has attained its maximum growth in height (4.6–4.8 cm.). The amino and amide nitrogen values for the axis increase throughout this period, while the protein content shows little change.

The nitrogen changes in the cotyledons are represented in figure 4. Total and protein nitrogen decrease simultaneously until the ninth day; the protein then increases somewhat, possibly at the expense of amide nitrogen, which decreases slightly at this time. After the fifth day the amino nitrogen content is constant.

Tables 3 and 4 give the data for the concentration of amino and amide nitrogen in the water of the tissues. For the axis, the amino nitrogen concentration is fairly constant, with the exception of the value on the fifth day. The other values are too irregular to be of much significance.

In all the experiments ammonia nitrogen never exceeded 2 per cent of the total, and in most cases was much less. The peptide nitrogen in the axis constituted about 2 per cent of the total nitrogen in both light and dark series. In the cotyledons of the dark series the peptide nitrogen constituted 10 per cent of the total nitrogen for the first 5 days, then decreased to less than 2 per cent by the ninth day. In the illuminated series the peptide nitrogen fell to less than 2 per cent of the total nitrogen by the fifth day.

Discussion

Bonnet (1), in a study of the nitrogen metabolism of *Lupinus luteus* in the very early stages of germination, reported significant increases in amino and amide nitrogen when the shoot just appeared (on the second day under the conditions reported in this paper). He gave no data on the subsequent changes occurring in axis and cotyledon.

McKie (3) studied the changes occurring in lupins grown in a soil-sand mixture up to 25 days. She reported a minimum value for protein nitrogen on the eighteenth day, and decreasing values for amino and amide nitrogen from the eighth day onward. The asparagine content rose to a maximum of 53 per cent of the total nitrogen on the eighteenth day under her conditions of culture and analysis, and in all experiments amino and amide nitrogen was very low as compared with pro-

tein and "insoluble" nitrogen. The relations of "protein" and "insoluble" nitrogen to each other and to the other nitrogenous constituents are usually ill-defined unless the nature of the insoluble fraction is known, which is generally not the case.

Figures 1 and 2 (dark series) show that the amino and amide nitrogen of the cotyledon changed but little after the fifth day, whereas the protein content continued to decrease until the ninth day; concomitant with this protein decrease, the amide and amino nitrogen contents of the axis increased in close parallelism, suggesting a quantitative relationship among these three constituents. It should be noted that after the ninth day the protein content of the cotyledon changed very little but the amino acids of the axis continued to increase, suggesting synthesis of this nitrogen fraction.

With regard to the plants grown in the light, it appeared that illumination either promoted protein synthesis or inhibited its hydrolysis after the ninth day, as the protein content of the cotyledon was at a higher level in the illuminated series as compared with the etiolated plants. A similar condition was found by VICKERY *et al.* (5) to occur in the excised tobacco leaf after 73 hours. From the ninth to the fifteenth day a reciprocal relation existed between amide and protein in the cotyledon. The "protein sparing" action of light may be due to the fact that the illuminated plant obtains its energy for growth by the oxidation of carbohydrate synthesized in the presence of light, whereas the etiolated plant hydrolyzes protein to supply some of the required energy, unless it has adequate carbohydrate reserves. It is possible that under the conditions of these experiments photosynthesis cannot supply carbohydrate for energy purposes until the ninth day.

Associated with the increased protein content of the illuminated plants were definite morphological differences; thus the etiolated plants had small yellow cotyledons and tall thin stems (8.5 cm. in 15 days) whereas the illuminated plants had large green cotyledons and short sturdy stems (4.7 cm. in 15 days). These relations indicate that longitudinal growth and protein synthesis are not necessarily associated, and that even a reciprocal relation may hold.

Attention has already been called to the constancy of the values for amide and amino nitrogen concentration in the water of the tissues. Although these concentrations are irregular, they are of the same order of magnitude for each constituent throughout the period studied. This suggests that a dynamic relation exists between protein hydrolysis and the concentration of the soluble hydrolytic products.

Summary

1. Data are presented on the values and distribution of the chief nitrogenous constituents of the lupin plant at progressive stages of germination.

2. Protein disappearing from the cotyledon was found to accumulate in the axis as amino acids and amides.

3. Plants grown in the dark were found to hydrolyze more protein and transport the products to the axis sooner than did plants grown in the light.

4. Longitudinal plant growth may take place to a marked extent with little or no change in the actual protein content of the elongating part.

5. The amino and amide nitrogen concentrations in the water of the lupin tissues remain at the same order of magnitude throughout the first 15 days of germination, suggesting a dynamic relation between these two nitrogen fractions and protein hydrolysis.

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STARCH HYDROLYSIS IN BEAN LEAVES FOLLOWING SPRAYING WITH ALPHA NAPHTHALENE ACETIC ACID EMULSION

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(WITH FOUR FIGURES)

Introduction

In previous investigations concerning the response of plants to applications of growth substances such as indoleacetic, naphthalene acetic, and indolebutyric acids, it was noted that starch in tissues near the treated region was hydrolyzed following treatment, and that although carbohydrate was in some cases mobilized in the treated region of stems, it did not occur as starch. The disappearance of starch from the region of bean and tomato stems treated with a mixture of indoleacetic acid and lanolin has been reported (**1, 3**). Other investigations have shown that starch was not present in the region of the stems of bean plants to which indoleacetic, naphthalene acetic, or indolebutyric acids were applied (**3, 4, 5, 6**). These responses suggested that the presence of these acids possibly increased the diastatic activity of enzymes in some cells that responded to treatment. In the present investigation a more direct study of the effect of some growth regulating substances on the rate at which starch was digested during a period of low light intensity or in darkness was made; also the rates of sugar accumulated in treated and control plants during a period of illumination were compared.

To facilitate the study, treated and control leaves of bean plants were grown: (*a*) in alternate periods of natural daylight and darkness, (*b*) in complete darkness, and (*c*) in natural daylight. The starch and sugar content of treated and control leaves under these various conditions was determined quantitatively.

Methods and preliminary experiments

The heart-shaped leaves of kidney bean plants, Calapproved variety, were used. The plants were grown in soil in 4-inch clay pots, an excess being planted so that uniform plants could be selected for each experiment. In experiments in which attached leaves were required, all portions of the plants above the second node were removed previous to treatment so as to accelerate the accumulation of carbohydrates in the primary leaves and to prevent further extension of the stem or production of additional leaves.

In preliminary experiments, detached leaves of bean plants were rooted in

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sand kept moist with complete nutrient, and exposed to natural daylight until they contained a relatively large amount of starch. Of some of these the entire upper surfaces were painted with a warm 2 per cent lanolin mixture of naphthalene acetic acid; of others only portions were painted in a similar way; still others were treated in the same manner with pure lanolin, as controls. One week later the chlorophyll was removed by means of hot alcohol and the leaf blades immersed in weak iodine solution. A strong positive starch test was observed in control leaves, while leaves treated with the lanolin-naphthalene acetic acid mixture showed in most cases a much less deposit of starch in the treated areas. In some few instances these treated areas gave no positive starch test. The results indicated that—other conditions being the same—more starch was hydrolyzed in areas of the leaves to which the lanolin-naphthalene acetic acid mixture had been applied than in those to which only lanolin was applied. Leaves still attached to the plants were treated and found to respond in a similar manner. Naphthalene acetic acid in a lanolin water emulsion was then tried and this was found to be a more suitable method of application than had been used. Application of an emulsion containing 1 per cent naphthalene acetic acid resulted in increased hydrolysis of starch in leaves without noticeable toxicity, and this concentration was chosen for further experimentation.

The following method for making emulsions was adopted. Two hundred and fifty mg. of alpha naphthalene acetic acid was thoroughly mixed in 25 gm. of melted lanolin. The mixture was added to 250 ml. of boiling water in which 1.25 gm. of laundry soap and 0.2 gm. of agar had been dissolved. The mixture was thoroughly stirred, cooled, and applied to the leaves by means of a spray, using a paper collar around each petiole, just below the leaf blade, to protect the remainder of the plant from the spray.

For chemical analysis, duplicate samples consisting of twenty-four leaves each were collected, dried at 80° C. in a well ventilated oven, and ground to 100 mesh. They were redried at 80° C. in a vacuum. The sugar, and the combined starch and dextrin contained in 1-gm. samples, were determined as previously described (5). Sucrose was hydrolyzed by means of invertase and the total amount of reducing and non-reducing sugars determined.

Experimental results

A series of three experiments was conducted. The object of the first experiment was to determine whether the starch, dextrin, and sugar content of sprayed leaves varied from that of control leaves when the plants were grown in alternate periods of natural daylight and darkness subsequent to treatment.

Plants having leaves 2–3 inches in width and containing appreciable amounts of starch, as shown by iodine tests, were used. Several hundred plants were

selected for size and uniformity and placed in rows on a greenhouse bench. The upper surfaces of the heart-shaped leaves of the plants in each alternate row were then sprayed, at 3:00 P.M. on a clear day, with 1 per cent naphthalene acetic acid emulsion. Leaves of plants in the remaining rows were sprayed on the upper surfaces with an emulsion of pure lanolin, to serve as controls. Initial samples were collected at random from both sprayed and control plants immediately after treatment. Samples were collected during the following night at 11:00 P.M.; and at 9:00 A.M., and again at 9:00 P.M.—following a dark cloudy day. Epinasty occurred within 90 minutes after treatment but otherwise there were no obvious differences between sprayed and control plants.

TABLE 1

SUGAR, STARCH, AND DEXTRIN CONTENT OF SPRAYED AND CONTROL LEAVES. SAMPLING BEGAN AT END OF A CLEAR DAY, AND CONTINUED DURING THE NIGHT AND FOLLOWING DAY, WHICH WAS DARK AND CLOUDY. FIGURES REPRESENT AVERAGE OF DUPLICATE DETERMINATIONS OF PERCENTAGE OF SUGAR, STARCH, AND DEXTRIN, EXPRESSED ON DRY WEIGHT BASIS

Time of sampling	Hours after spraying	Percentage			
		Sugar		Starch and dextrin	
		Control	Treated	Control	Treated
3:00 P.M.	0*	2.4	2.4	3.8	3.8
11:00 P.M.	8	2.1	2.8	2.8	2.7
9:00 A.M.	18	1.6	3.2	1.5	0.5
9:00 P.M.	30	1.0	1.6	1.8	0.0

* Sampled at time of treatment.

Table 1 shows that the starch and dextrin content of sprayed leaves decreased until finally the leaves contained an immeasurable amount of these substances, while only approximately one-half the starch and dextrin was hydrolyzed in control leaves during the same interval of time. The difference in starch and dextrin content of sprayed and control leaves was not noticeable until at least 8 hours after treatment.

Iodometric tests made at frequent intervals during the experiment likewise showed marked decrease in starch content of sprayed leaves after approximately the fifteenth hour following treatment.

The sugar content of sprayed leaves increased appreciably above that of controls following application of the acid, but later decreased rapidly as starch digestion continued, and finally reached approximately the same percentage concentration as that of controls at the time the experiment was concluded.

A second experiment was performed in which the digestion of starch in sprayed leaves was studied during a prolonged period of darkness, in order to eliminate any effects due to photosynthesis. The plants used in this instance were older than those of the previous experiment and the starch content of the heart-shaped leaves at the beginning of the experiment was greater. To facilitate handling, the plants were placed on trucks so that they could be easily transferred to a darkroom immediately following the spraying. The upper surfaces of the primary leaves of half the plants were sprayed as previously with 1 per cent naphthalene acetic acid emulsion and the leaves of the remaining plants were sprayed with emulsion of pure lanolin. The plants were placed in the dark immediately after

FIGS. 1–3.—Starch content of representative leaves as shown by iodine test: fig. 1, at time of treatment; fig. 2, after 19 hours of darkness following spraying with pure lanolin emulsion; fig. 3, after 19 hours of darkness following spraying with 1 per cent naphthalene acetic acid–lanolin emulsion.

treatment and initial samples for chemical analysis were collected at random. Subsequent samples were collected as previously described at intervals during the following 48-hour period. The temperature to which the plants were exposed during the experiment varied between 70° and 75° F.

The percentage of starch and dextrin in both sprayed and control leaves increased slightly for several hours after the plants were removed from natural daylight of high intensity and placed in darkness (table 2; fig. 4). Subsequent to the initial increase, the starch and dextrin contents of both sprayed and control leaves decreased at approximately the same rate until the fourteenth hour, after which time starch digestion progressed at a greater rate in sprayed than in control leaves. Similar results were shown by means of the iodine test, which was made repeatedly during the experiment (figs. 1–3).

As in the previous experiment, the percentage of sugar in leaves to which the acid emulsion was applied increased appreciably several hours after treatment, but remained at a relatively high level for a period of only a few hours, then decreased to approximately the same percentage as that of controls.

These data corroborate those of the previous experiment, in that the use of 1 per cent naphthalene acetic acid emulsion accelerated the rate of starch digestion in the leaves, and the increased rate of hydrolysis was associated with a relatively high sugar content that persisted until most of the starch and dextrin was digested.

TABLE 2

SUGAR, STARCH, AND DEXTRIN CONTENT OF SPRAYED AND CONTROL LEAVES. PLANTS CONTAINING RELATIVELY LARGE AMOUNTS OF SUGAR, STARCH, AND DEXTRIN WERE TREATED, IMMEDIATELY PLACED IN THE DARK, AND SAMPLES COLLECTED. FIGURES REPRESENT AVERAGE OF DUPLICATE DETERMINATIONS OF PERCENTAGE OF SUGAR, STARCH, AND DEXTRIN EXPRESSED ON DRY WEIGHT BASIS

Hours after spraying	Percentage			
	Sugar		Starch and dextrin	
	Control	Treated	Control	Treated
0*	2.1	2.1	9.7	9.7
3	1.6	1.6	10.6	10.3
6	1.5	1.5	10.3	9.5
10	1.3	2.3	9.4	9.2
14	1.4	2.5	9.3	9.5
20	1.1	2.6	8.5	6.2
30	1.1	1.6	6.8	3.8
48	0.9	0.8	4.3	2.4

* Sampled at time of treatment.

Data from additional experiments, in which indoleacetic acid and phenylacetic acid were substituted individually for naphthalene acetic acid, showed that these substances also accelerated the rate of starch hydrolysis. In these experiments the effect of these substances on the starch content of leaves was demonstrated by means of iodine tests.

A third experiment was conducted to study the effect of sprayed naphthalene acetic acid emulsion on the accumulation of sugars, starch, and dextrin in the heart-shaped leaves of beans. After the terminal buds of relatively young plants had been removed at the second node, the plants were placed in darkness until the leaves were depleted of starch as shown by iodine tests. The leaves of half the plants were then sprayed on the upper surfaces with 1 per cent naphthalene

acetic acid emulsion as previously described, and the remaining half with lanolin emulsion. The sprayed and control plants were immediately transferred from

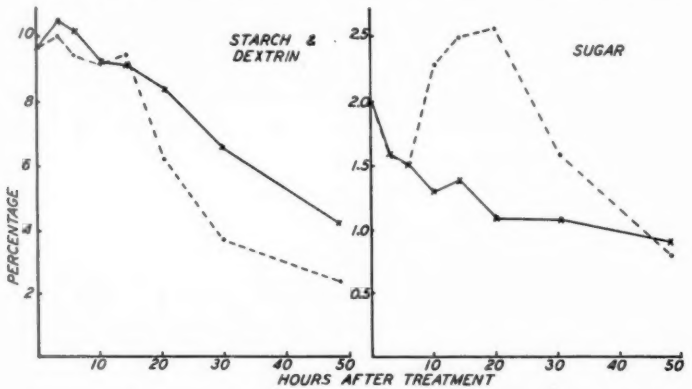

FIG. 4.—Percentage of sugar, starch, and dextrin in primary leaves of bean plants sprayed with 1 per cent naphthalene acetic acid emulsion and kept in the dark for 48 hours (broken lines), as compared with comparable leaves sprayed with pure emulsion (solid lines). Percentage calculated on dry weight basis.

TABLE 3

SUGAR, STARCH, AND DEXTRIN CONTENT OF SPRAYED AND CONTROL LEAVES. PLANTS PLACED IN DARK UNTIL SUGAR AND STARCH CONTENT WAS DEPLETED, THEN EXPOSED TO NATURAL DAYLIGHT. FIGURES REPRESENT AVERAGE OF DUPLICATE DETERMINATIONS OF PERCENTAGE OF SUGAR, STARCH, AND DEXTRIN EXPRESSED ON DRY WEIGHT BASIS

Time of sampling	Hours after spraying	Percentage			
		Sugar		Starch and dextrin	
		Control	Treated	Control	Treated
7:00 A.M.	0*	0.34	0.34	0.0	0.0
8:00 A.M.	1	0.9	0.9	0.0	0.0
9:00 A.M.	2	1.7	1.6	0.0	0.0
10:00 A.M.	3	3.2	2.5	Trace	Trace
11:00 A.M.	4	4.2	2.9	2.6	1.6
12 NOON	5	4.9	3.0	2.6	2.3
1:00 P.M.	6	5.9	3.4	6.1	4.4
2:00 P.M.	7	6.3	3.5	7.6	4.6
3:00 P.M.	8	5.5	3.6	8.0	5.9
5:00 P.M.	10	5.7	3.8	9.3	6.4

* Sampled at time of treatment.

the darkroom to the greenhouse, where they were arranged in alternate rows. They were illuminated by means of natural daylight, and duplicate samples were

collected from sprayed and control leaves at the end of each hour for a period of 10 hours. The light intensity increased from 750 foot-candles at 7:00 A.M. to 2000–3000 during the middle of the day, then decreased to approximately 500 foot-candles at 5:00 P.M. On several occasions during the day the sun shone brightly and the intensity reached a maximum of approximately 10,000 foot-candles during short intervals. The temperature was 75° F. at the beginning of the experiment and increased during the morning, reaching a maximum of 90°–92° during the interval between 1:00 and 5:00 P.M.

The rate of sugar accumulation was approximately equal in sprayed and control leaves during the 2 hours immediately following treatment, but after this interval control leaves accumulated more sugars than did the sprayed leaves (table 3). The accumulation of starch was likewise less in sprayed than in control leaves.

Discussion

When certain concentrations of mixtures of naphthalene acetic acid and lanolin are applied to the stems of actively growing bean plants, many of the cells composing certain regions of the stem—such as the endodermis, phloem, cambium, rays, and pith—manifest greatly increased meristematic activity (**1, 2, 3**). This is true both for embryonic cells and for those which show advanced stages of maturation in differentiation, wall thickening, vacuolization, and food storage. If, as in the latter type of cells, they contain starch, one of the first readily detectable changes is the disappearance of the starch grains, accompanied by a general increase in the density of the cytoplasm, sometimes an enlargement of the entire cell, and very often subsequent cell division. There is in effect a restoration of the embryonic condition in many of the cells which have already differentiated. It is probable that the reserve carbohydrates, dextrin, starch, and hemicellulose are hydrolyzed to less complex forms of carbohydrate in cells which respond to treatment with naphthalene acetic acid, and that such forms may be more rapidly and more readily metabolized.

From the data presented it is evident that naphthalene acetic acid increased the rate of starch digestion in the leaves of beans to which it was applied. The results indicate that the acid may have affected diastatic activity indirectly, as 10–15 hours elapsed following treatment before an appreciable difference was noted between the starch and dextrin contents of the sprayed and control leaves. Associated with more rapid digestion of starch was a marked increase in the sugar content of the sprayed leaves. This increase was observed in sprayed leaves kept in darkness, and when the digestion of starch neared completion their sugar content decreased and finally approached that of control leaves. It is therefore probable that this temporary accumulation of sugars in sprayed leaves was, partially at least, caused by the increased rate at which complex forms of carbohydrates were hydrolyzed owing to application of the naphthalene acetic acid.

It is also probable that the application of 1 per cent naphthalene acetic acid retarded the accumulation of carbohydrate in leaves, as both the sugar and starch contents of sprayed leaves were less, during a period of illumination, than that of controls. Further studies may determine whether this sugar content of sprayed leaves resulted from increased respiration, transport, or lower rate of photosynthesis.

Summary

1. A 1 per cent naphthalene acetic acid–lanolin and water emulsion was sprayed on the upper surfaces of attached bean leaves. The digestion of starch and accumulation of sugars during a period of darkness or low light intensity and the rate of accumulation of sugar and starch during a period of illumination were studied by means of quantitative chemical determinations made at intervals following treatment.

2. The percentage of starch and dextrin in leaves sprayed with naphthalene acetic acid emulsion and placed in darkness decreased more rapidly than did that of control leaves. Noticeable differences, however, were not apparent for several hours after treatment. The percentage of sugar in leaves sprayed with the acid emulsion and placed in darkness increased during that period following treatment when starch digestion was most rapid, but later decreased as the starch reserve was depleted, finally reaching a value approximately equal to that of untreated leaves.

3. Leaves that had been depleted of sugars, starch, and dextrin by being kept in the dark and subsequently sprayed with naphthalene acetic acid emulsion and then placed in natural daylight accumulated less sugar, starch, and dextrin during the period of illumination than did comparable untreated leaves.

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EVOLUTIONARY SIGNIFICANCE OF RING POROSITY IN WOODY ANGIOSPERMS

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(WITH TWELVE FIGURES)

Introduction

With the increasing use of wood anatomy in phylogenetic investigations, it is important to check carefully the principal lines of specialization in xylem structure. One of the best known features of gross anatomy is the arrangement of the elements, especially the large spring vessels in deciduous trees. It is generally considered that ring porosity represents a high degree of such specialization; but there has been disagreement as to whether such a structural modification has any marked evolutionary significance or represents merely an ecological adaptation. The present study attempted to settle this question and also to determine the direction and nature of such an evolutionary change with respect to angiosperm origin.

Recent work by HUBER (**15**), PRIESTLEY (**28**), and LODEWICK (**26**) on the conduction of water and the nature of cambial activity indicates that this structural difference has definite physiological implications. Because of this it is generally considered that ring porosity represents a specialization from the diffuse porous condition, yet there appears to have been no serious morphological study of this viewpoint. HUBER believes—on purely physiological evidence—that the ring porous condition is a phylogenetically advanced feature allowing rapid conduction of water in the early spring followed by formation of the more drought-resistant summer wood.

The only other discussions of the evolutionary origin of the two types that were found in the literature are by GROOM (**14**), FROST (**9**), and BAILEY (**2**). The first of these deals with *Quercus* alone. GROOM points out that three main courses of evolution are possible in deriving the annual ring of the oaks. These he gives as follows:

"1. Regressive from a typical ring with marked pore-zone.
2. Divergent from a ring with a feebly marked pore-zone.
3. Progressive from a ring with no indication of a pore-zone."

GROOM does not commit himself as to which of these possibilities represents the actual evolutionary course. The fact that he worked with only the mature wood may have led to his hesitancy, for this genus exhibits almost all the possible types of vessel arrangement.

On the other hand, FROST states: "Ring porous woods tend to have larger vessels than diffuse porous woods. There is a high correlation between the diffuse porous condition and the presence of scalariform perforations. As one might expect, the distribution of primitive vessels follows the distribution of tracheids (that is, diffuse or more or less evenly spaced throughout the xylem)" (**9**).

No data were given by FROST to support this conclusion, and a check made on the tables of BAILEY and TUPPER (**4**)—the basis of FROST's work—showed no such correlation. This reconsideration is discussed later in this paper. The general conclusion of FROST is still tenable, however, that the primitive type of vessel arrangement was diffuse.

BAILEY (**2**) also states that the ring porous condition is highly specialized. "The typical ring porous condition arises apparently when plants which have undergone characteristic structural modifications in certain tropical or subtropical environments are subjected to cold winters or to alternating very dry and wet seasons. In other words this is closely associated with the acquisition of a pronounced resting period and the commencement of the deciduous habit. It is significant that certain species of a widely dispersed genus may be ring porous and others diffuse porous, depending upon their phytogeographical distribution." He regards this as evidence of parallel lines of specialization.

These generalizations seem to be plausible, but little concrete evidence was advanced to support them. Pure speculation can also make a possible case for the opposite point of view—that the diffuse porous type is a reduction from the ring condition. The Amentiferae are considered primitive by many systematists; this group has a number of ring porous species. The Gnetales have been postulated as close to the point of angiosperm origin; the most generally primitive member of this family (*Ephedra*) is ring porous (fig. 1).

As SINNOTT and BAILEY (**38**) have shown, the tree form is to be regarded as the primitive type for angiosperms. If this is so, then the question of the structural pattern of the wood becomes important in any attempt at a reconstruction of the ancestral race. If neither is primitive, one is forced to assume a marked polyphyletic origin of the present groups. While this possibility must be logically allowed, it is contrary to the trend of modern concepts of morphology.

In the present investigation two methods of attack are employed, one qualitative and the other more quantitative in nature. The first is based on the principles developed by JEFFREY and his students. As they have been extensively described in the literature (**16, 17, 3, 35**), a brief outline will suffice here. The theory of recapitulation is used as a basis for postulating that there can exist "conservative" regions in the plant body that will retain more primitive features of organization than will the others. Moreover, under the stimulus of certain types of injury reversion may occur to the phylogenetically original morphological feature. Thus

in attacking the present problem, the anatomy of the first annual ring, node, root, and reproductive axis has been investigated in addition to the study of seedling structure. Some attention has also been given to the effect of traumatism upon ring porosity.

Within the last 20 years there has been developed another promising method of studying evolutionary trends in specializations of wood anatomy. The foundations of this were principally laid by the work of BAILEY and TUPPER (4). FROST (9, 10) has given a discussion of the theory and application of this method.

The technique depends on the previous establishment of an undoubtedly primitive anatomical feature. This is then associated with the feature to be investigated to determine the extent and direction of the correlation between the occurrence of both features in the various species. A high positive correlation would indicate that the feature studied is relatively primitive. If a similar characteristic shows a more negative correlation with the established primitive feature, it would be considered more advanced and the line of specialization could then be read from the first to the second characteristic.

FROST applied this method in a study of the development of the vessel. He used as his original primitive characteristic the length of the tracheary elements. BAILEY and TUPPER had already shown that the most advanced element was the shortest one. FROST associated the length of the vessel with the type of perforation and the end wall. He found a high degree of correlation between the primitive conditions of length and scalariform perforation. From data such as these he concluded that the vessel was derived from the scalariform tracheid and that the wood of the homoxylous angiosperms (*Trochodendron, Drimys*, etc.) "is unquestionably very primitive."

These same methods were used by KRIBS (23, 24) in studying the salient lines of specialization of the wood rays and wood parenchyma of dicotyledons. He used as his basis the vessel types of FROST. According to KRIBS' conclusions the specialized types are the homogeneous ray and the vasicentric abundant distribution of wood parenchyma.

It is generally agreed (16, 11) that the fibrous wood elements have been derived from tracheids by a reduction of the bordered pit to the simpler nonbordered condition. A wood with its fibers having definite borders on the pits is regarded as showing a more primitive condition with respect to this feature.

In this paper the presence of the ring porous vessel arrangement was associated with each of these advanced wood structures, and the degree of correlation determined. Unfortunately the extensive material necessary for a really statistically sound investigation was not obtainable. The data, however, were already listed in a number of reference works on wood anatomy (29, 30, 44, 39, 12, 27, 33, 40). The paper of BAILEY and TUPPER (4) was of particular value in this connection. In

view of these extensive data on the wood anatomy of the various species, it was decided that good indication of the correlation trends could be obtained by a thorough search through the literature and the statistical use of the accumulated data.

One important feature of the present application of this method is to be noted. It is well known to students of wood anatomy that the ring porous type is almost anomalous in consideration of the overwhelming preponderance of diffuse porous woods in the flora of the world. As will be emphasized later, occurrence of the ring condition is restricted to a geographical region—the North Temperate zone. In consideration of the effect of environment on plant distribution and evolution, it is regarded as unsound to attempt to correlate on a world-wide basis the various lines of specialization with the development of the ring porous condition. Such studies must be made only with plants of the North Temperate zone. The necessity for such restriction is obvious since it is manifestly impossible for specializations in wood structure occurring in the tropics to have any relationship to ring porosity, when that type does not and cannot exist under the climatic conditions characteristic of tropical regions. The presence of a diffuse arrangement in a tropical species is no criterion of a primitive wood.

There is no indication that FROST (**9**) has considered this qualification in the use of the data. This may explain the inaccuracy of his statement regarding "the high correlation between the diffuse arrangement of vessels and the scalariform condition of the end wall."

Material and methods

The material consisted of the wood of different organs of various ages and locations from about sixty different species, representing eleven families.[1] The writer is responsible for all identifications except for the material from the Sonderegger and United States Soil Conservation nurseries. Almost all the collecting was done in the neighborhood of New Brunswick, with the exception of some material from South Jersey and that of *Quercus virginiana* which was obtained from Florida through the kindness of Dr. M. A. CHRYSLER. I am indebted to Dr. FORREST SHREVE for the specimens of *Ephedra trifurca* from Arizona.

Sections were made of the woody cylinder of the different organs of plants of various ages. In general, vigorous, well grown material was selected. In most cases only cross sections were made, and therefore the term vessel as used in this paper strictly includes any tracheary element of diameter greater than about 0.05 mm. No serious error is introduced by this convention, since the diagnostic vessels of a ring porous type are much larger than this (at least five times), and tracheids are generally smaller.

[1] Details of material and results will be found in table 1 of the thesis submitted in partial fulfillment of the Master of Science degree, Rutgers University. 1938.

Experimental work

A. Evidence from comparative anatomy

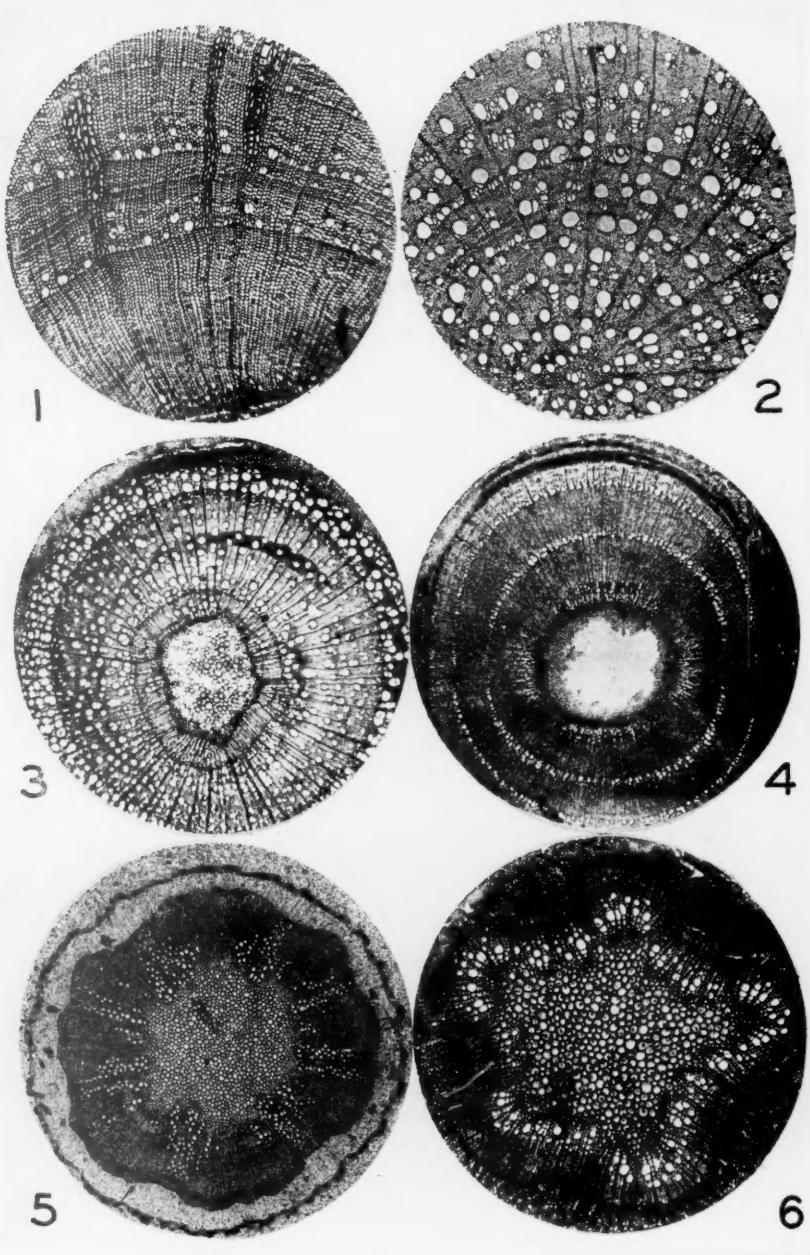
1. Root.—Roots of all the investigated genera were examined and found to be diffuse (fig. 2). Riedl (31) found this condition to be so constant that the use of this feature was not possible in the construction of a systematic key for the identification of root wood. Wounded root wood of *Ulmus americana* showed only the diffuse condition in all parts adjacent to and at the point of injury. This is only negative evidence, of course, and is not of theoretical importance since reversions do not necessarily accompany all cases of injury.

2. First annual ring and node.—All diffuse porous species examined showed no essential difference in vessel distribution in the first annual ring as compared with the adult wood. In sections just below the node, however, there was a tendency toward grouping of the larger vessels in the metaxylem and early secondary xylem associated with the "leaf supply" to the leaves immediately above. This was especially evident in *Acer platanoides* and *Fagus americana* (figs. 4, 7, 10). As this was also seen in sections of many of the ring porous types and seemed to be a special condition associated with the leaves, no special recapitulatory significance was attached to it. But as the metaxylem and early secondary xylem of the leaf trace are continued basipetally in the progressively older portions of the annual ring, it is evident that continuous production of leaves throughout most of the annual increment of growth develops a diffuse condition of the vessels. Likewise any reduction of leaf production or restriction to an early spring phase with continued wood formation in the summer will lead to a markedly ring type.

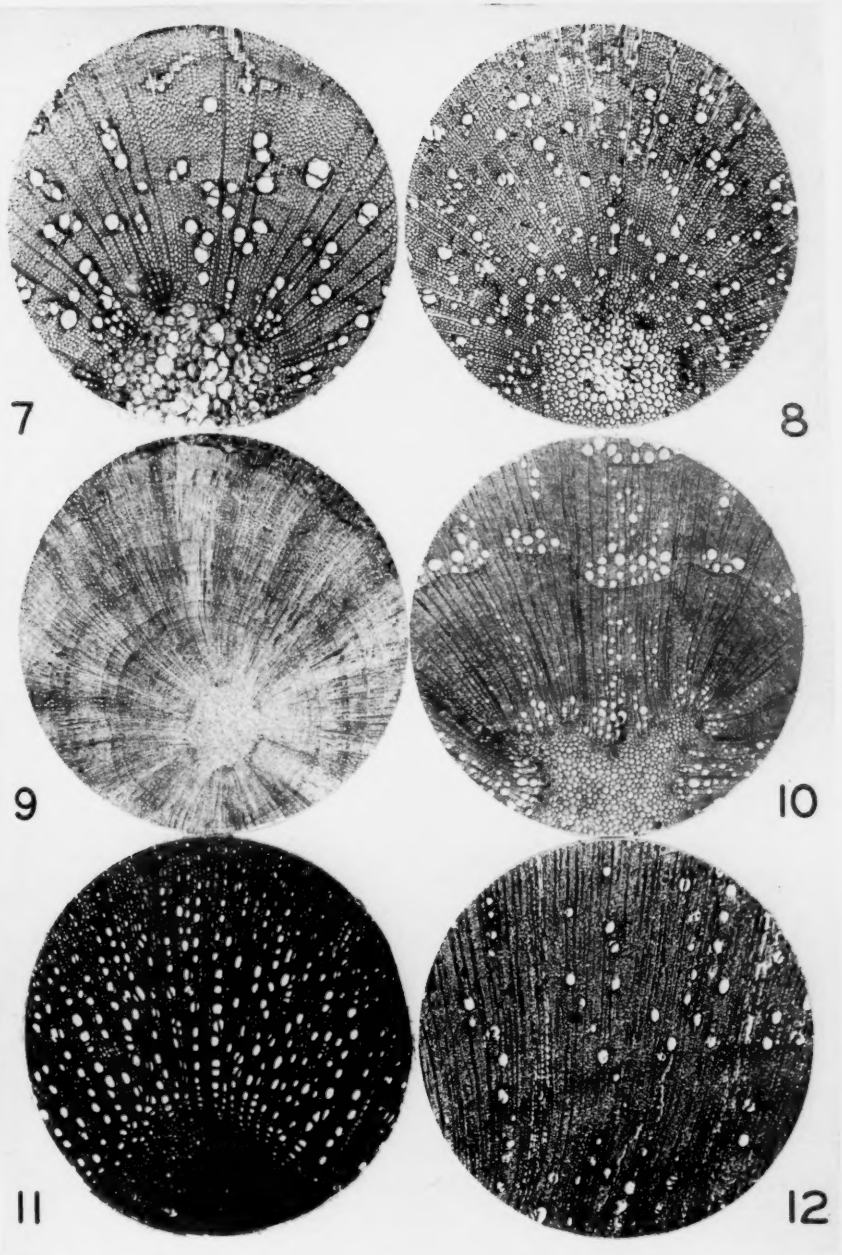
In almost all genera which are normally ring porous in the adult wood, the first year's growth was diffuse. In some instances this condition persisted through a number of subsequent annual rings before the adult condition was established (figs. 3, 9). In all probability this recapitulatory rate can be accelerated or made slower according to the environmental conditions present.

In some species of *Quercus* this tendency was poorly evident or could be considered as on the borderline. Examples of this are *Q. imbricata*, *Q. marilandica*, *Q. phellos*, and *Q. prinoides*. *Q. prinus*, on the other hand, gives evidence of a shorter recapitulation of this feature, being amost characteristically ring porous in the first annual ring of a twig from an adult tree. In these cases, however, the concave pith "depression" showed markedly greater diffusion of pores than the xylem opposite the pith arms. At the node the leaf gap, of course, showed only parenchyma.

From observation of serial sections through two successive nodes and study of young twigs stripped of their bark, it was evident that the fluted appearance of the twig was associated with the phyllotaxy.



FIGS. 1–6.—Fig. 1, *Ephedra trifurca*, branch showing ring porous arrangement. Fig. 2, *Ulmus americana*, normal root. Fig. 3, *Morus* sp., twig showing slow recapitulation. Fig. 4, *Ailanthus glandulosa*, young twig, rapid recapitulation. Fig. 5, *Quercus borealis*, seedling above hypocotyl. Fig. 6, *Q. alba*, seedling upper stem showing ring porosity.



FIGS. 7–12.—Fig. 7, *Robinia pseudo acacia*, hypocotyl near node. Fig. 8, *Celtis occidentalis*, vigorous seedling stem. Fig. 9, *Quercus ilicifolia*, seedling stem 10 years old, diffuse porous. Fig. 10, same, adult branch ring porous. Fig. 11, *Castanea dentata*, peduncle, diffuse porous. Fig. 12, *Quercus* (*velutina?*), portion of traumatic tissue magnified to show diffuse arrangement of pores.

JEFFREY (16) calls the fluted appearance of the oak stem a "depression or retardation of growth," yet there is evidently a different set of factors at work here. The "depressed" segment is of exactly the same width as the rest of the annual ring (fig. 10). If anything, there is acceleration of growth, for in the latter rings of a branch the fluted appearance is lost, indicating that the growth rates must be slightly faster to correct for the earlier fluting. This effect is clearly the result of the type of cambial activity and primary meristematic growth associated with formation of the leaves. PRIESTLEY (28) has shown that the direction of xylem differentiation is basipetal. Thus in the formation of the vascular cylinder the earliest differentiation will be in that portion which will later be the leaf supply. Initiation of the rest of the stele will be associated with the leaves of the successively higher nodes. Thus retardation of both time and position of origin will be produced, but no differences in absolute growth rate will be evident.

The influence of phyllotaxy on the vascular anlage can be seen clearly in the figures of LANGDON (25). The vascular supply to the leaf was found by her to be formed before the leaves themselves were well differentiated in the seedling buds of *Quercus*.

3. SEEDLING ANATOMY.—Seedlings of eighteen species of ring porous dicotyledons were examined. In all cases the plants were at least one year old, and of good vigor compared with others of the same lot. Eleven species were from the nurseries of the Soil Conservation Service at New Brunswick and were selected as "vigorous." Poorly developed specimens were also examined to check the findings, but no significant differences were obtained. In the case of *Ulmus americana*, over twenty seedlings of varied growth status were compared. The results obtained were as follows:

(a) All seedlings showed hypocotyls that were diffuse porous (fig. 7).

(b) A similar condition was found at the cotyledonary node.

(c) All first year seedling stems were likewise diffuse (figs. 5, 8, 9), with the exception of those examined of the genus *Quercus*. These showed a definite tendency toward the ring porous condition near the apex of the stem (fig. 6), although half way below this the condition was markedly diffuse (fig. 5).

(d) The two-year-old seedling of *Fraxinus americana* of exceptional vigor was likewise diffuse the first year and ring the seond. In *F. pennsylvanica*, a normal seedling of one year's growth showed the typical diffuse condition even at the apex of the stem.

4. PEDUNCLE.—In consideration of the well known conservatism of the reproductive organs, an examination was made of the floral axis of those plants characterized by the formation of a considerable cylinder of secondary wood. Members of the genus *Quercus* are particularly favorable for this purpose; especially so are the members of its subgenus *Erythrobalanus*, which retain their acorns for two

years, thus forming two annual rings in the floral axis. Herbarium material was used to insure identification of the species. Other genera used are noted below.

The three species of *Lepidobalanus* showed only a diffuse annual growth. This was especially marked in the large peduncle of *Prinus* but was also clear in *alba* and *bicolor*. All the species of *Erythrobalanus* were diffuse porous in both annual rings except *phellos*, which had a tendency toward ring porosity near the main axis. The region near the acorn base was decidedly diffuse porous in both rings. The peduncle of *Castanea dentata* was markedly diffuse porous (fig. 11). The floral axis of *Ailanthus glandulosa* was slightly ring porous near the abscission region and diffuse near the inflorescence.

It is evident that the data from the floral axis agree with the previous evidence in establishing the primitive nature of the diffuse porous condition.

5. WOUNDED STEMS.—Close examination of a number of cases of severe wounding in young oak branches showed definite signs of reversion to the diffuse condition as a result of the hypertrophy produced. This stimulating effect was especially marked in the sector formed directly from the injured portion and in the wound cap proper. Figure 12 shows the condition of the stem in the region adjacent to the wound.

The observed effects were an increase in the diameter of the summer vessels and reduction in size of the spring pores that were formed after wounding. A plausible explanation of this result may lie in the new growth situations present in the wounded region. The spring growth may have been retarded and the transition to the period of summer growth made less sudden by the effects of wounding followed by hypertrophy. The question awaits study of the physiological factors involved.[2] Whatever these factors may be, it is evident that in this case there is definite reversion to the ancestral type of vessel arrangement. It may be possible that the conditions of hypertrophy are analogous to the more favorable climatic conditions of earlier geological history.

As the physiological agents are so poorly understood and so important in this type of phylogenetic investigation, no great reliance is placed on this evidence. It is of significance only in relation to the more reliable data that have been obtained. The results are recorded principally to show that these data do not conflict with the other findings. It is to be noted, however, that BAILEY (1) does not record such traumatic effects in his study of wounded oak stems.

B. CORRELATION STUDIES

A study of the correlation between the presence of ring porosity and certain other advanced features of wood anatomy has been made. The results are given in table 1. The families considered are listed in tables 10 and 11 of RECORD (30).

[2] A recent paper (21) on day length is of interest in this connection.

The results of this investigation show in general such high correlation that they may be regarded as conclusive, even though the data are admittedly incomplete with regard to exotic genera.

There is perfect correlation between the ring porous condition and the most advanced type of vessel wall perforation, in so far as genera are concerned. There is probably a negative correlation in the case of the indigenous diffuse porous genera. The correlation in regard to the families with ring porous representatives is almost as perfect, but no such correlation can be demonstrated for the families whose indigenous representatives are exclusively diffuse porous. In view of

TABLE 1

STATISTICAL STUDY OF SALIENT LINES OF STRUCTURAL SPECIALIZATION AMONG RING POROUS AND DIFFUSE POROUS GENERA AND FAMILIES

Class	Vessel perforation		Pitting on wood fiber		Dominant ray type		Wood parenchyma distribution	
	Simple	Scalariform	Simple	Bordered	Heterogeneous	Homogeneous	Paratracheal	Other types
Indigenous ring porous genera‡	53	0	27	6	18	14	52	1
Indigenous diffuse porous genera†	9	18	3	27	23	12	2	18
Indigenous ring porous families‡	20	4*	22	3	16	4	13	1
Indigenous diffuse porous families†	24 (Record)	20	9	21	15	3	0	25
	23 (Bailey)	23						
All diffuse porous families in entire world†	59	37	75	80	.....	.....	.....	.....

* Mostly simple perforations. † Exclusively diffuse type. ‡ Some ring porous members present.

FROST'S statement (9, p. 74), there is special significance in the world-wide data on families whose tree members are exclusively diffuse porous. If anything, the figures show a weak correlation between diffuse porosity and advanced vessel type.

Two of the other features associated with ring porosity in this study agree with the preceding findings in regard to the advanced nature of the ring porous type. These are the nature of the pitting of the wood fiber wall and the distribution of wood parenchyma. The other, the type of ray, shows nothing of phylogenetic significance in an attempt to correlate either of the main types with the vessel pattern. It is also to be noted that the heterogeneous ray is the dominant type in temperate North America.[3]

[3] The numerous genera of the Leguminosae are of the homogeneous ray type. On the basis of the families, the heterogeneous ray is the dominant type in the ring porous families (table 1).

Discussion

Ring porosity is restricted to a definite geological region, that of the North Temperate zone. Members of the same genus, and even supposedly the same species, may be ring porous in one region (for example, North America) while farther south they show the diffuse condition exclusively. The Leguminosae, for example, have about sixteen genera of trees in North America and all are ring porous. There are more than 125 genera in tropical South America and all are diffuse porous. The genera *Quercus*, *Ulmus*, *Celtus*, and *Trema* also show this feature clearly (**29, 44**). The Southern Hemisphere, even in the temperate regions, has no listed ring porous trees.

The evidence from the fossil record, incomplete as it is, shows certain pertinent facts regarding the possible origin of the ring porous type. JEFFREY (**16**) has shown that the late Carboniferous represents the beginning of the annual ring, with the geographical origin of this feature in the boreal regions.

The climatic conditions associated with the formation of the annual growth habit cannot be restricted to the Northern Hemisphere, for SAHNI (**34**) and BARNARD (**5**) have described fossil woods with annual rings from the early Tertiary of the southeastern regions of the world. GOTHAN (**13**) found two species of *Lauroxylon* and certain coniferous woods from the Upper Cretaceous and Tertiary of the Antarctic that show definite rings. The earliest known woods of a definite angiosperm nature show no signs of a ring porous arrangement, even though well developed vessels are present (**37, 41, 42**).

While the fossil wood of the early Tertiary is mainly diffuse porous, the Miocene is characterized by the occurrence of the transitional type. FELIX (**7**), KNOWLTON (**20**), and JEFFREY and CHRYSLER (**18**) have described such types. The succeeding epochs show the development of all the present day genera of trees, although some of them had a more extended range in the interglacial periods (**6**).

There is abundant evidence to indicate that the ring porous type of vessel arrangement represents an evolutionary advance from the diffuse distribution. This conclusion is based on the agreement of the results of investigations using such different methods of attack as that of the comparative morphology of JEFFREY's school and the statistical approach of BAILEY and his students. Thus it has been shown that the evidence from the seedling, first annual ring, root, and the reproductive axis points definitely to the primitive nature of the diffuse porous type. The evidence from the traumatic phenomena is to be regarded as inconclusive in itself, although definite indications of reversion to the primitive arrangement are to be found in severely wounded stems of *Quercus* (*velutina?*).

A high degree of correlation has been shown to exist between ring porosity and

the presence of structural features whose advanced nature has been universally conceded. These are the simple perforation of the end wall of the vessel element, the paratracheal distribution of wood parenchyma, and the presence of only simple pits on the wood fibers. This fact is strikingly evident in the lower families having ring porous members. In such cases the diffuse porous genera and species of the North Temperate zone tend to have the primitive features, while the ring porous ones are almost entirely specialized.

In the Juglandaceae, *Carya*, which is the characteristically ring porous genus, has pitting on the wood fibers with such faint borders that SOLEREDER considers this a genus with the simple pit feature and distinguishes it from the other genera on this basis. *Platycarya*, a monotypic genus of Asia, is of a less marked ring porous nature, and has definite bordered pits on its wood fibers (**22**). The other genera are diffuse porous and show the bordered condition. Only simple perforations are found in the first two genera, and also in the widespread genus *Juglans*, which shows a tendency toward ring porosity in two species (*J. cinerea* and *J. nigra*). The other two genera, *Engelhardtia* and *Alfaroa*, are of restricted range, diffuse porous, and with some to many vessel elements with scalariform perforations.

In considering the phylogenetic distribution of the ring porous feature, one is impressed with the fact, suggested by BAILEY (**2**), that so many important families of different positions in the ENGLER-PRANTL system of classification have ring porous representatives. This would seem to indicate either a polyphyletic origin of this feature or an early appearance before there was marked diversification of the primitive angiosperms. The finding of wood like *Laurinoxylon* from the beginning of the Tertiary lends support to the latter possibility.

WIELAND (**43**) has called attention to the hypothetical derivation of the angiosperm wood from the Cycadeoids of the Cretaceous and Jurassic through a homoxylous type as an intermediary. Whatever may be the status of the Cycadeoids in this question, the vessel-less angiosperms have assumed increasing importance in tracing angiosperm origin. The wood of *Pataloxylon scalariforme* (**5**) is especially interesting in that its sole conducting elements were large scalariform tracheids, while the other species *P. porosum* had both large tracheids and scalariform vessels. SAHNI (**36**) found a fossil wood (*Homoxylon rajmahalense*) with marked affinity to the living homoxylous Magnoliaceae (*Drimys*, *Tetracentron*, etc.). This fossil was "probably Jurassic." Similar woods have been described from the early Tertiary of Greenland and Germany (**43**). It should be noted, however, that JEFFREY and COLE (**19**) do not regard the vessel-less members of the Magnoliaceae as primitive, but as reduced. More recent anatomical work (**9, 32**) has tended to agree with the fossil evidence in regard to the primitive nature of the homoxylous wood.

The other Magnoliaceae are closely related to these genera and are likewise considered to be of early origin. This, however, does not preclude some cenogenetic evolution in successful genera like *Magnolia*. Through the courtesy of Dr. WILLIAM E. ROEVER, access was given to his abundant material of wood of eleven species of the Magnoliaceae. The only species showing any signs of a tendency toward ring porosity was *M. acuminata*. In a private communication, ROEVER stated that he had likewise noticed this tendency. This fact is especially interesting since earlier (**32**) he regarded the species as having in general the most advanced wood structure of those investigated.

Thus it is entirely within the realm of scientific possibility that the ring porous type may have developed as the result of a change in the climatic conditions of the late Cretaceous. An increasing aridity would first produce the more efficient *Fagoxylon* type of wood with its wide vessels. The development of a winter season followed by spring thaw and summer drought would produce marked seasonal inequality in the available moisture supply. This condition is that occurring in the present oak-hickory subclimax forest (**8**). The beech-maple climax is of a more mesophytic nature, however, and more closely akin to the supposed Cretaceous climate. This factor was probably at work in producing the abundant Miocene and Pliocene ring porous flora.

Those families which developed their taxonomic entity after this specialization of pore arrangement could have retained ring porosity as a genetical possibility, requiring only the proper habitat for its expression. Thus it is seen that those members of the higher families (Bignoniaceae, Papilionaceae, Scrophulariceae, etc.) which have tree forms in the North Temperate zone are exclusively of the true ring porous type.

The diffuse porous members of the most advanced families are not therefore to be regarded necessarily as of more primitive origin than the ring porous members of the lower groups, or even of their own group. Ring porosity represents only one of the many possible lines of specialization that could be followed in developing a plant body that would be successful in a given environment. Conditions in the tropics are obviously not conducive to the formation of the ring arrangement, and this feature is to be regarded as of no phylogenetic significance in those regions The temperate zones, however, afford an opportunity for the study of the development of this type of arrangement.

Summary

1. The problem of the evolutionary significance of the ring porous type of vessel arrangement has been investigated. Two methods were employed, one based on that of JEFFREY and the other on the correlation method of BAILEY and his

students. Both methods gave identical results, indicating that they are both valid in this type of problem.

2. Data from the anatomy of the seedling, first annual ring, root, and reproductive axis show that diffuse porosity is the more primitive condition for trees which are ring porous in the adult stem.

3. A high degree of correlation has been shown to exist between ring porosity and the presence of structural features whose advanced nature has been generally conceded. These are the simple perforation of the vessel segment end wall, the paratracheal distribution of wood parenchyma, and the presence of only simple pits on the wood fibers. The ray type does not show this correlation. From these data it is concluded that ring porosity is an advanced feature.

4. The evidence indicates that this specialization took place early in angiosperm history and affords no proof for the existence of parallel lines of evolution. Ring porosity has probably developed as a response to the climatic conditions characteristic of the North Temperate zone. These conditions are now peculiar to a wide but delimited region of the world, which also represents the modern range of the specialized type. Any morphological considerations of this structural specialization are valid only within the limits of that range.

The writer acknowledges his gratitude to Professor M. A. CHRYSLER, whose guidance and criticism have aided immeasurably in the development of this work.

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RESPONSE OF THE PEANUT PLANT TO INOCULATION WITH RHIZOBIA, WITH SPECIAL REFERENCE TO MORPHOLOGICAL DEVELOPMENT OF THE NODULES

O. N. ALLEN AND ETHEL K. ALLEN

(WITH ELEVEN FIGURES)

Introduction

Although nodules of the peanut, *Arachis hypogaea* L., were noted by POITEAU (27) as early as 1853, only occasional mention of the plant was made in nodulation studies until 1917, when BURRILL and HANSEN (6) placed it in the cowpea cross-inoculation group. Only recently has attention been given the plant in nitrogen-fixation studies. In the present investigation two phases of the rhizobium-leguminous plant complex have been studied: (*a*) the infective and effective abilities of various strains of rhizobia upon the growth of the plant, and (*b*) the histology and cytology of the nodules, the development of which apparently has not been heretofore described in detail.

Greenhouse studies

Two commonly cultivated varieties, the Spanish and the Jumbo, were used in the tests. Plants of the former variety tend to grow erect, bear fruit around the base of a single stem, and can withstand considerable moisture; those of the latter are the trailing type and have greater xerophytic tendencies. Prior to planting, the shelled seeds were surface-sterilized in 1:1000 mercuric chloride for several minutes, washed in sterile water heated to 56°–60° C., rinsed in cold, sterile distilled water, and dried overnight at room temperature.

The inocula consisted of fifty-nine strains of rhizobia isolated from a variety of leguminous plants. The infective and effective abilities of these strains for other leguminous species have been noted previously (1).

The sterilized seeds were planted with the micropyle downward in sterile quartz sand. Ten-day-old cultures of the organisms in asparagus-potato mannitol broth were applied in the usual manner and the seeds covered with sterile sand. Germination, as evidenced by swelling of the seeds and their protrusion through the sand, usually occurred about the fourth day, although the plumule did not emerge until about the tenth day. By that time an appreciable root system had been formed. The growing period was 10–12 weeks, or until a considerable number of gynophores on each plant had penetrated the sand and were in the process of setting seed. The plants were watered as often as necessary with sterile distilled water and received Crone's solution weekly. The results of the greenhouse tests

have been based on a series of replicated experiments in which the strains of rhizobia were run in triplicate against both varieties. At the time of harvest the roots were examined in trays of water to facilitate detection of the different types of nodules.

The results are shown in table 1. The data obtained with the two varieties have not been tabulated separately, since the differences in types of nodules produced and the responses in growth were identical. Nodules usually made their appearance about the time the third set of leaves was forming, while their effect upon growth of the plant was not apparent until the fifth week.

TABLE 1

RESPONSE OF PEANUT PLANT TO INOCULATION BY VARIOUS STRAINS OF RHIZOBIA ISOLATED FROM TROPICAL LEGUMINOUS PLANTS

LUXURIANT GROWTH, DARK-GREEN FOLIAGE, MODERATELY LARGE NODULES ON CENTRAL ROOT SYSTEM	POOR STUNTED GROWTH, YELLOW TO YELLOWISH-GREEN FOLIAGE, INNUMERABLE SMALL NODULES THROUGHOUT ROOT SYSTEM
GROUP A: GOOD STRAINS	GROUP C: INEFFECTIVE STRAINS
Indigofera suffruticosa 7–4, 7–5	Phaseolus lathyroides 1–4, 1–5
Samanea saman 13–5, 13–7	Cassia mimosoides 5–2, 5–3
Arachis hypogaea 19–6, 19–7, 19–8	Andira inermis 20–1, 20–2
Desmodium barbatum 22–1, 22–2*	Vigna marina 27–1, 27–2
Stylosanthes guianensis 23–1, 23–3	Canavalia campylocarpa 32–6, 32–7
Erythrina indica 36–1, 36–4	Enterolobium cyclocarpum 34–7, 34–10
Albizzia lebbek 39–1, 39–3	Lonchocarpus domingensis 74–1, 74–5
Alysicarpus vaginalis 77–3, 77–6	Piscidia erythrina 75–4
GROUP B: MODERATELY GOOD STRAINS	Derris microphylla 86–2, 86–3
Acacia koa 15–3, 15–4	Tephrosia candida 107–1, 107–3
Cassia chamaecrista 58–6	Hymenaea coubaril 100–1, 100–2
Piscidia erythrina 75–1	Inga laurina 108–1, 108–2
Parkia africana 89–1, 89–2	Pongamia pinnata 87–2, 87–4
Cytisus scoparius 2	Vigna sinensis 602, 603, 604
Glycine hispida 518	Glycine hispida 534, 500
Crotalaria spectabilis 9–5	Lupinus arboreus 1, 3, 7

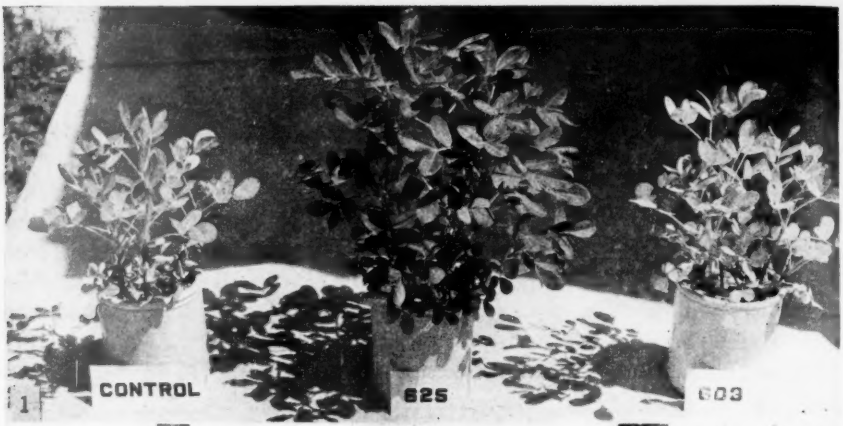
* Now strain 625 of the Wisconsin collection.

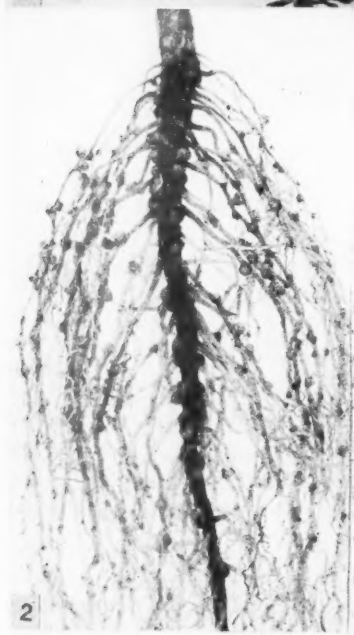
In general the nodules occurred singly, remained simple and spherical, and were similar to the lupine type, inasmuch as there was always a rather broad basal connection with the root, indicative of continued pericambial activity. All nodules (figs. 2, 3), irrespective of their size and source of inocula, were located in the root axils. This arrangement was conspicuous since the root system of this species consists of a central taproot, along which the lateral roots arise in an orderly alignment of four vertical rows, owing to the tetrarch pattern of the xylem. On no occasion were nodules found randomly dispersed along the roots in the arcs between

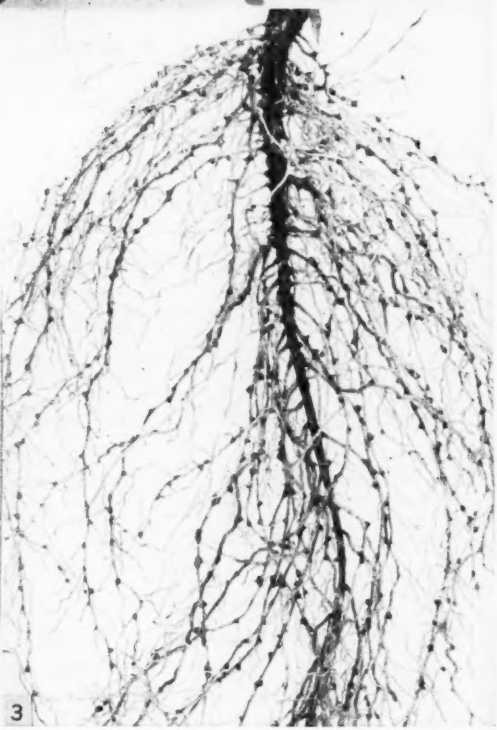
the protoxylem points, nor did they occur in the regions opposite the protoxylem points where lateral roots had not emerged.

Good agreement was obtained between the growth response of the plants and the size and location of the nodules (figs. 1–3). The strains of rhizobia could easily be divided into three groups on the basis of effectiveness. Although the plants proved rather susceptible to nodulation, as evidenced by the fact that each of the strains produced nodules, only twenty-six strains benefited plant growth. Seventeen of these, as designated in group A, markedly stimulated plant growth, while nine others were beneficial to a lesser extent. The plants nodulated by these latter strains (group B) were significantly better than those of the control and group C series, but differed appreciably from those in group A in regard to dry weights and percentages of fixed nitrogen. There were no differences, however, in the types of nodulation produced by the effective strains (groups A and B), since comparatively large nodules, more or less concentrated on the central upper portion of the root system, were common on all the plants showing green foliage and enhanced growth. The strains comprising group C were definitely non-beneficial, as shown by a stunted, sickly, yellow-green condition of the plants and the occurrence of small nodules which tended to be inconspicuous in the axils of the secondary and tertiary rootlets. The nodules produced by these strains were analogous to those obtained by CARROLL (7) on peanut plants when strains of rhizobia of *Lespedeza*, soybean, cowpea, and velvet bean were used as inocula. In a few experiments conducted during the summer months, plants nodulated by certain of the strains in group C were inferior to those in the control or uninoculated series.

In addition to the small ineffective nodules common to the roots of the peanut, a proliferation comparable in external appearance to the collar type of nodule (41) was frequently noted. This condition is faintly visible in figure 3. To the naked eye these nodose formations were similar to young nodules, although when immersed in water they appeared as bulbous conical swellings completely surrounding the basal portions of the fibrous rootlets. Where two small nodules occurred simultaneously, one in the upper and the other in the lower axil of the rootlet, it was somewhat difficult to distinguish them from the collar or nodose condition, although a morphological differentiation was usually possible by means of a hand lens. All attempts to isolate rhizobia from these swellings were unsuccessful. Histological examination showed them to be merely hypertrophied parenchymatous tissue containing heavy deposits of starch. Since these formations showed no evidence of infection, did not contain bacteria at any stage of development, bore no anatomical similarities to nodules, and were found only on uninoculated plants and those nodulated by the ineffective strains (group C), it is thought they were abnormal proliferations related in some way to nitrogen deficiency. It is likely that a confused interpretation of these abnormal proliferations







FIGS. 1–3.—Fig. 1, response of peanut plant to inoculation with rhizobia: left, no inoculation; center, inoculated with strain 625 (strain 22-2 of University of Hawaii collection), isolated from *Desmodium barbatum* (L.) Benth. and Oerst.; right, inoculated with strain 603, isolated from *Phaseolus lunatus* L. Fig. 2, nodule formation when inoculated with beneficial strain 625. Fig. 3, same when inoculated with non-beneficial strain 603.

in relation to non-beneficial nodules is responsible for the conclusions of RAJU (29) and RAJAGOPALAN (28) that the size and location of nodules on the peanut are not correlated with the effective abilities of the rhizobia.

Histology and cytology of the nodule

MODES OF DEVELOPMENT AMONG THE LEGUMINOSAE

Although the exact mechanism by which the bacteria enter the root is not known, it is generally agreed that the root hairs constitute the normal avenues of infection. It is likely that the rhizobia secrete an enzyme which enables them to penetrate the hair wall; however, cellulose-splitting enzymes have not been detected in cultures of the organisms. Nevertheless, when the rhizobia come in contact with the root hairs, the hair wall is apparently softened or dissolved, curvature of the hair results, and the rhizobia proceed through the hair in a threadlike manner until the basal epidermal cell is reached. At this point the thread frequently becomes multiple, and their penetration into the cortex is accompanied by rapid division of parenchymatous cells. Differentiation within the hypertrophied mass of cells gives rise to the nodule tissues. Other avenues of infection have been known to occur. As early as 1888, BRÉAL (4) was successful in securing infection by piercing the roots of seedlings with a needle which had been dipped in a culture of rhizobia. Other investigators (6) have shown the possibility of rhizobia entering directly through epidermal cells of young root tips, especially where the plants are cultured in artificial media (such as agar) which ordinarily suppress root hair production.

The extent of penetration of the infection threads and the tissues primarily stimulated are important factors in determining the site of nodule origin. THORNTON'S (34) suggestion that the exact location may differ according to the species of host plant seems rather pertinent. In nodules described as exogenous, infection has been limited to the parenchymatous cells of the cortex, and the endodermis and those tissues within the central stele of the root are not directly concerned in their formation other than in the production of the vascular system necessary in the later stages of nodule development. Development of nodules of this type has been described in detail by FRED, BALDWIN, and MCCOY (12), THORNTON (34), and BIEBERDORF (3). On the other hand, VAN TIEGHEM and DOULIOT (38), PARATORE (24), PEIRCE (25), WHITING (40), and TERBY (33) have described nodules of endogenous origin, a condition wherein the nodules have originated by division of pericyclic cells, usually opposite protoxylem points. Nodules of this type have provoked many complications, owing to their similarities to lateral root formation. In general the data offered in support of endogenous origin have not been so convincing or so complete as those presented for exogenous origin; consequently

many phases of their development have not been made clear. It is hoped that certain of these aspects will be clarified by the following observations of endogenous nodule formation.

Material and methods

Most of the plant material used in the histological study was obtained from the afore-mentioned culture experiments. Occasionally seedlings were cultured in moisture dishes when material was necessary for the detection of root hairs and young nodule stages. In the later phases of the study, field material was obtained for the purpose of comparing the results obtained from sand-grown and soil-grown plants. Fixation of the root material was made at various intervals of time so that successive stages of development could be noted. Frequently entire root systems were fixed in the hope of finding the primary stages of infection. Care was always taken to see that the nodules were not detached, in order that histological sections could be made through the juncture.

Four fixatives were used: Bensley's, Flemming's medium, Wallin's, and formalin-acetic-alcohol. The first three were equally satisfactory in the detection of embryonic nodular and rootlet development, since the osmic acid blackened those areas having concentrated protoplasm and chromatin materials. Use of the microscope (low power objective) or hand lens made it possible to detect these areas prior to their emergence through the root cortex. Flemming's fixative, modified either by decreasing the amount of acetic acid to about 10 drops per 100 cc. or by omitting it entirely, was the most satisfactory solution for routine use. Bensley and Wallin's fixatives were especially useful in differentiating mitochondria and bacteria; while the formalin acetic alcohol, the least satisfactory, was used only when a comparative study of the anatomical features of the tissue was primarily desired. All material was fixed 24–48 hours (depending upon the size of the plant portions), washed, dehydrated, imbedded in 58° paraffin, and cut in serial sections 3–10 μ thick.

One of the most satisfactory techniques for the study of cellular contents was the placing of surface-sterilized nodules in drops of sterile water on clean glass slides and cutting them in halves. One-half of a nodule was then placed in a certain fixing solution, while the characteristics of the other half were studied by stained smears or in wet mounts. When the major portions of a nodule were treated by different fixatives, the cellular contents in the drop of water or thin free-hand sections through the center of the nodule served for examination of the fresh material. Various staining methods were used to bring out certain details. Flemming's triple, Heidenhain's iron-alum haematoxylin with and without counterstains, Bensley's stain for mitochondria, and MacCallum's modification of the

Gram stain were found satisfactory. Heidenhain's haematoxylin without a counterstain was usually preferred, since uniformity resulted because of the more sensitive control of the staining procedure.

Discussion and results of histological study

No significant differences were noted in the histology and the cytology of the nodules produced by the effective and ineffective strains of rhizobia. Similarly there were no differences in the structure of the nodules produced on plants of the two varieties. Consequently the following observations pertain in general to the development of nodules, irrespective of these factors.

Mode of infection

It became apparent in the initial phases of the histological study that infection of the roots by rhizobia was complicated by several conditions uncommon to other members of the Leguminosae heretofore studied. Although the roots of the peanut are of the taproot type, the distribution and occurrence of the root hairs were unusual. Instead of the hairs being uniformly common and abundant in the regions of elongation of the central and lateral roots, they were extremely rare. When lateral or tip hairs were observed they were few in number and arose at random from widely separated epidermal cells. These findings were contrary to expectation, since it was assumed that the plant would normally have abundant root hair formation, owing to its semi-xerophytic tendency and ability to grow well in warm, loose sandy soil. Such an unusual condition probably accounts for the reports of Richter (according to Reed, **30**) and Pettit (**26**) that hairs are entirely absent on peanut roots. On the other hand, tufted clusters or rosettes of hairs, such as noted by Waldron (**39**) and Reed (**30**), were frequently found in the root axils. These hairs were less fragile than those common on the lateral surfaces of other leguminous roots, had moderately thick cell walls, and averaged 2–4 mm. in length. They were found on all plants of more than 2 weeks' growth and under all conditions of culture.

Little explanation is given for the presence of the hairs in this unique arrangement. Waldron (**39**) found their growth stimulated by high temperature and humidity, but none of the environmental factors he employed explained their presence in the root junctions and the absence of the normally expected lateral type. His conclusion, that lateral hairs are absent because of the loose arrangement of the epidermal and cortex cells, which "seem readily to fall apart like so many poorly cemented bricks becoming loosened," was not confirmed in this study. The root systems of all the plants were white, well developed, and showed no tendency to slough off the cortex layer of cells. It is probable that root hair production was stimulated in the axils as a result of tissue injury and retarded epi-

dermal growth caused by rootlet emergence. In nodulated plants further stimulation of the cells in the axillary locations was undoubtedly provided by subsequent formation of the nodules which, as will be shown later, arose from within these regions and pushed outward through the ruptured tissues. These observations are of interest since SNOW (31) and others have shown that root hair production is commonly stimulated at the site of wounding and especially in those areas where elongation of the epidermal cells has been arrested owing to abnormal swellings. Inasmuch as lateral root hairs occurred infrequently throughout the various methods of plant culture, it is believed that their presence in whorls or tufts around the base of the side roots is perhaps one of inherent, normal nature with this species, rather than a result of the particular environment.

Since root hairs and nodules were found only in axillary regions, it might be expected in the light of previous data (12) that formation of the latter resulted from infection of the former. Such was not shown to be the case. In a series of experiments in which the development of nodules and root hairs was closely followed, none of the data were compatible with the idea that the root hairs participated in the infection process. In the majority of instances where nodules and root hairs occurred in the same axils, hair formation was simultaneous with, or subsequent to, the first indications of nodule development. As the nodules produced by the effective strains enlarged, there was a tendency for the hairs to become less conspicuous; in many cases they were entirely absent. On the other hand, in the presence of the non-beneficial nodules the hairs tended to be persistent. There was close agreement between the presence of the whorled tufted hairs in the axils and the "nodose" condition on the plants showing nitrogen lack. Frequently the hairs had their origin in the epidermal cells of these hypertrophied areas. In several experiments seedlings showing a prevalence of axillary hairs were immersed in suspensions of rhizobia and in their filtrates. On no occasion was curvature or the presence of bacteria observed in the root hairs. This was also true when the randomly scattered lateral hairs were examined. Apparently the rhizobia lack the ability to effect entrance through the hair wall.

The mechanism by which the rhizobia enter the roots becomes a matter of conjecture, since definite data in the affirmative were not obtained. BEIJERINCK (2) and MCCOY (19) have suggested the probability of infection through the ruptured tissue of the root at the time of rootlet emergence. It appears likely that the peanut plant is an example, since nodules did not occur in regions other than the root axils. Further evidence validating cortical splits as avenues of infection was provided by the production of nodules in the axils of adventitious roots emanating from stems which had been covered with soil above leaf branches. On no occasion were nodules formed on the adventitious roots per se, nor did root hairs occur in their junction with the stem. The nodules produced in these axils were identical in

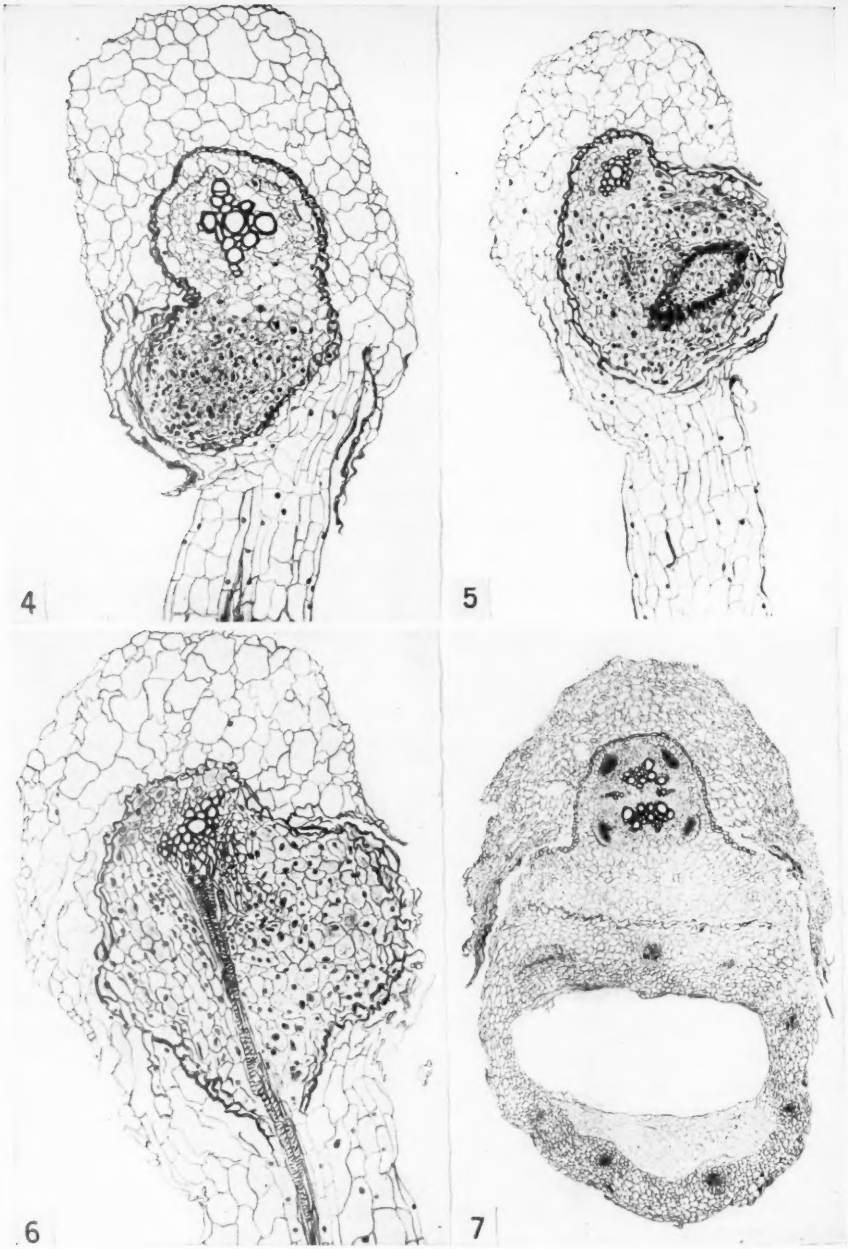
every respect with those formed on the root systems. Various chemical, physical, and mechanical factors common to sand and soil culture undoubtedly facilitate infection through the ruptured tissues.

Differentiation of nodule tissues

The earliest evidence of nodule formation was a meristematic activity in the pericyclic cells adjacent and in juxtaposition to the same protoxylem strand from which a rootlet had emerged. Students agree that lateral roots also arise in this layer of cells. Fixed preparations of root systems of young inoculated plants showed nodule meristems in the axillary positions about 2 weeks after rootlet emergence. The fact that these areas were not apparent prior to emergence of the rootlet is further indication that the ruptured tissues provided entrance for the rhizobia. The meristems were characterized by small, irregular-shaped, thin-walled cells containing dense cytoplasm, a large nucleus with a fine, evenly granular karyolymph, and one prominent homogeneous nucleolus. As the infected area enlarged by rapid cell division, the densely packed cells tended to push outward and form a conelike proliferation projecting away from the central stele of the mother root. Throughout this stage growth activity remained within the endodermis, which expanded accordingly. Figure 4 shows the location of this meristem during early stages of nodule development.

Occasional mitotic figures and evidences of recent cell division were frequently discernible in the cells surrounding the center zone of growth. The activity of these cells did not appear at first to be affected by the presence of rhizobia within the cytoplasm, although, as the cells attained their maximum size and became filled with bacteria, mitosis diminished and finally ceased. Small vacuoles were not common in the young infected cells; on the other hand, large central vacuoles were prevalent in mature stages. The nucleus and the nucleolus persisted in the cytoplasm of the infected cells until the latter became filled with rhizobia and by-products of the symbiosis. With the production of a central vacuole and increased tension in the cytoplasm, the nucleus was pushed to one side, where it assumed various irregular, crescent, or amoeboid shapes prior to its disintegration.

Dissemination of the rhizobia, or the spread of infection, within the bacteroidal area occurred during mitotic division of meristematic cells, as previously shown by Lechtova-Trnka (15). The mode of spread, however, did not appear to be dependent upon the polar attraction of the rhizobia, as described by Milovidov (21) in the lupine nodule. In general the process was analogous to the condition in the bean nodule (19), inasmuch as the rhizobia were more or less passively divided with the cytoplasm as the cell plate was formed between the daughter cells. The efficiency of this mode of bacterial spread was evidenced by the fact that in mature nodules the central bacteroidal area appeared as a solid mass of densely



FIGS. 4–7.—Fig. 4, cross section of primary root and nodule, and longisection through lateral rootlet, showing embryonic nodule (meristematic area) inclosed within endodermis. Fig. 5, later stage showing exit of nodule from root parenchyma, formation of cambial ring, and differentiation of tissue. Fig. 6, tangential section through same nodule and median longisection through lateral rootlet showing close relationship between rootlet and axillary nodule. Fig. 7, cross section of primary root and old nodule prior to complete disintegration. Note the eight vascular bundles in nodule cortex, remnants of bacteroidal area below hollow center, and cortical sheath of mother root.

filled cells not unduly hypertrophied as a result of infection. Single or clustered uninfected cells were not common, although zones—each consisting of two or three rows of empty parenchymatous cells—occasionally extended from the periphery into the center of the bacteroidal area. It is likely that these were cells not infected when initial cell division occurred. On no occasion were infection threads or zoogloeal strands noted as agents in the transmission of organisms to cells within the bacteroidal area.

In the embryonic nodule the primary mass of undifferentiated meristem showed cell division taking place in all planes. This resulted in a bowl-shaped or spherical proliferation, in contrast to the type of growth described by SPRATT (**32**) and others (**3, 25, 34**), where a meristematic cap at the distal end of the nodule causes it to increase in length and become cylindrical. The endodermis continued to envelop the proliferation (fig. 4). The nodule mass usually made its exit from the root tissues about one week after infection. At this time differentiation within the embryonic area was evidenced by the production of a cambial sphere which in cross section appeared as a ring of small, heavily stained meristematic peripheral cells several layers in thickness. This ring (fig. 5) functioned by producing two kinds of parenchymatous cells: to the outside—loosely packed, irregular-shaped cells containing little cytoplasm which served as nodule cortex; and to the inside—cells containing considerable cytoplasm which comprised the bacteroidal tissue. As growth and enlargement of the cells in the bacteroidal area took place there was consequent enlargement of the cambial sphere. During the initial stages of development of the bacteroidal area the cells were characterized by a dense cytoplasm, occasional vacuoles, central location of the nucleus, and inclusion of plastid-like bodies.

In regard to emergence, the peanut nodule simulated a rootlet in its ability to push or digest its way through the ruptured tissues of the mother root. In this respect it differed markedly from the nodules heretofore described on other leguminous plants. In all material examined the fragments of the mother cortex were retained loosely, in the manner of a partial sheath around the base of the emerged nodule (fig. 7). The fact that the nodule lacks a cortical covering provided by the mother root tissue becomes an important characteristic, since nodules of the exogenous type always remain inclosed within a layer of root cortex. About the time the nodule effected its exit through the root parenchyma, a periderm layer was formed along the exposed periphery from the collapsed and compressed cells of the nodule cortex. Since in roots the first true periderm commonly arises in the outer layers of the pericycle (**10**), its presence as a peripheral layer of the nodule serves as further evidence of its endogenous or pericyclic origin.

It was not until the nodule became macroscopically visible outside the root cor-

tex that vascular connection between the xylem and phloem of the root and nodule was evident. Development of the vascular tissues in the peanut nodules is apparently later than in nodules of cortical origin. Recently BIEBERDORF (3) has shown that conductive tissue in the soybean nodule may become noticeable soon after the rhizobia have penetrated the first two layers of the cortical parenchyma. The delay in vascular formation in the peanut nodule is probably accounted for by the inclusion of the meristematic area within the endodermis, where transportation of the nutrients and by-products between the nodule and the central vascular system of the root is facilitated by absorption and osmosis. The first evidence of the vascular system was a small meristematic area in the nodule cortex about midway between the cambial ring and the protoxylem strand (fig. 5). As division of the cells continued, the new walls were laid down more or less parallel to the radius of the root, and the xylem and phloem elements became differentiated in a tetrarch arrangement. These elements connected at right angles with the protoxylem strand, from which the nodule and the lateral root originated. On no occasion could vascular strands in a single nodule be traced to more than one primary xylem group, such as described in nodules of *Medicago* and *Vicia* (5, 36). The major conducting unit of the vascular system emanating from the central stele of the root encircled the nodule by means of four branches which developed at right angles to one another from the four protoxylem points at the nodule base. These branches subsequently divided, since eight vascular bundles were frequently seen in cross-section preparations of the mature nodule (fig. 7). These observations are in agreement with those of SPRATT (32), who was among the first to show a relationship between the xylem arrangement in the stele of the root and the vascular branching in the nodule.

Nodules have commonly been classified on the basis of morphology. In those characterized as compound, elongated, or cylindrical, growth always results from an apical or cap meristem. Thus as the nodule mass enlarges, the basal connection with the root tends to remain constant and thereby appears constricted. In consequence PEIRCE (25) assumed that as growth continued, the vascular connection between the nodule and the root became limited in function because of lack of secondary thickening at the nodule base, whereas the demands upon the conductive system tended to augment. He concluded that in such nodules the vascular system became inadequate and effected more rapid degeneration and death of the nodule cells. In comparison, peanut nodules occur singly and remain simple spherical formations. A broad basal connection with the root is maintained as a result of the prolonged cambial activity. In all the material examined the vascular connections between the nodules and the roots appeared adequate and normal until degeneration of the bacteroidal area was in an advanced stage.

Bacteria, mitochondria, and other inclusions of bacteroidal cells

Despite the fact that nodule tissues provide an excellent opportunity for studying the effect of the microorganisms upon the plant cells, and vice versa, it is not always possible to follow the changes occurring in the infected cells. In some instances difficulty arises from the rapidity with which the changes take place; in others, from the inability of methods to make clear the intricate transformations. In the peanut nodule the absence of infection threads or zoogloeal strands and the marked density of the cytoplasm in the small meristematic cells made it difficult to study the preliminary changes taking place in the infected cells of the bacteroidal area.

In general, fixed tissue preparations did not afford an acceptable medium for study of the rhizobia. Occasionally short, rod-shaped bacterial bodies, averaging 1 μ by 0.5 μ, were discernible along the periphery of newly infected cells, but the morphology and identity of these forms became less definite in later stages. Typical Gram negative rhizobia were always easily demonstrated in stained smears or wet mounts of a crushed nodule suspension. The young or physiologically active forms stained uniformly with weak carbol fuchsin (Ziehl formula diluted 1–10 with water) and other ordinary methods. With aqueous solutions of the anilin dyes the older or mature forms of the rhizobia appeared slightly swollen and showed uneven or banded areas within the bacterial cytoplasm. These banded areas alternated with one to three refractile bodies per cell, shown by the alpha-naphthol-paraphenylenediamine test of Dietrich and Liebermeister (**9**) to be fat inclusions. These findings are in agreement with those of Lewis (**17**), who demonstrated the fatty nature of the refractile inclusions in bacteroids of other strains of rhizobia.

Mitochondria were conspicuous in the young meristematic cells prior to formation of the cambial ring, although as the bacteroidal area became more mature, they were obscured by the other cellular contents. Their presence is generally interpreted as evidence of cellular stimulation caused by infection, or of an increased activity of the cells resulting from active division. Nodule tissues, fixed and stained, provided the only medium for examination of these bodies, since it was impossible to demonstrate them in wet mounts and stained smears owing to their fragility. Bensley's technique showed mitochondria in young infected cells of the bacteroidal area as reddish spherical bodies measuring about 0.5 μ in diameter against the cytoplasmic background counterstained by methyl green. Heidenhain's stain showed less differentiation, owing partly to simulation of the fat inclusions of the rhizobia to mitochondria, since both retained the haematoxylin more densely than the remainder of the bacterial cell.

The most conspicuous inclusions of the infected cells in the bacteroidal area

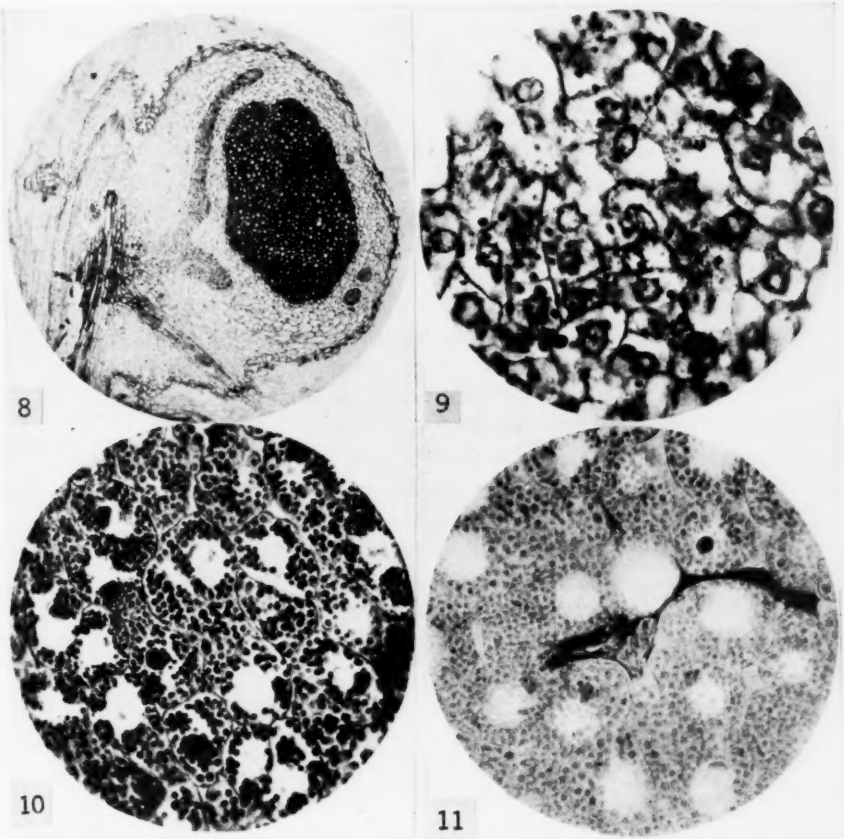
were spherical, plastid-like bodies which averaged 3 μ in diameter. These bodies first appeared singly and dispersed throughout the young infected cells of the centermost region of the nodule mass (fig. 9). On no occasion were they found in the meristematic cells, in the nodule cortex, or in the root tissues. As the cells in the bacteroidal area enlarged and became mature, the number of these spherules per infected cell increased rapidly. Frequently more than fifty such bodies appeared in cross section of a single cell. They stained heavily and occupied much of the cell space between the central vacuole and the cell wall (fig. 10). By the time differentiation of the tissue within the nodule was complete, the spherical bodies were so numerous that the mitochondria and rod-shaped bacteria could not be recognized with certainty. Apparently LECHTOVA-TRNKA (**15**) observed the same phenomenon, although she interpreted these bodies as large spherical forms of the rhizobia which had evolved from the rod-shaped stage. She assumed that the change in morphology was so rapid that all the rod forms in the cells were transformed into spheres at approximately the same time.

Various microchemical tests and staining methods were employed to identify these inclusions. In fresh, unstained wet mounts the bodies were refractive, colorless, and homogeneous. Reduced light or dark-field illumination showed the bulk of each body inclosed by a thin membrane and frequently containing several granules or one angular central granule in the ground matrix. LECHTOVA-TRNKA (**15**) suggested a likeness of these granular centers to nuclei, inasmuch as they occupied an appropriate position in the spherical forms which she assumed to be rhizobia. In general the spheres occurred singly, although a diplo arrangement was occasionally noted; however, when slight pressure was applied to the coverslip preparation, the joined bodies always separated with ease. The inability of these bodies to multiply was evidenced by no increase in numbers when sealed wet mounts were examined over a period of several weeks. Similarly there was no tendency toward disintegration or changes in form and size under these conditions. On the other hand, the number of rhizobia in the same suspensions was greatly augmented.

The inclusions gave negative reactions to tests specific for starch, glycogen, cellulose, pectin, tannin, and resins. The supposition that they were fat globules or involution forms of rhizobia undergoing fatty degeneration was not verified by the use of (*a*) Herxheimer's alcoholic-acetone solution of Sudan III in conjunction with a methylene blue counterstain, (*b*) Eisenberg's iodine-basic-fuchsin, (*c*) Dietrich and Liebermeister's naphthol blue method, and (*d*) Michaelis' Sudan IV. On the other hand, the rhizobia in the same microscopic fields showed positive fat tests, thereby providing a control on the technique. The bodies were unchanged after treatment with acetone, ether, and chloroform.

The spherical bodies from the fresh nodule material were insoluble and slightly swollen in dilute acetic acid. Their proteinaceous nature was established by weakly

positive reactions with the xanthoproteic test and Millon's reagent. Pronounced positive results were obtained in all tests with Krasser's methods as outlined by ZIMMERMANN (42) and ECKERSON (11). The fundamental mass of the spherical



FIGS. 8–11.—Fig. 8, longisection of mature nodule showing junction of vascular system of nodule and that of lateral rootlet with primary stele. Fig. 9, young bacteroidal cells showing rhizobia and beginning of aleurone formation. Fig. 10, bacteroidal area of mature nodule showing cells packed with aleurone. Fig. 11, same of old nodule showing dissolution of cell walls, disintegration of aleurone, and rhizobia packed in intercellular spaces.

bodies stained dark red with yellow crystalloid centers when thin sections of fresh nodule material or dried smears of crushed nodule suspensions were flooded with an alcoholic solution of picric acid and stained with alcoholic eosin. Similarly, the amorphous ground substance stained blue and the crystalloid center appeared

yellow-green after being treated in an alcoholic solution of nigrosin and picric acid. In consequence, these bodies were identified as aleurone or protein grains.

A rather marked affinity for a number of staining solutions was shown by the aleurone in smear as well as in tissue preparations. Variations were noted in the ability of the apparently young and old bodies to take the counterstain of the Gram method (Kopeloff and Beerman modification), but there was never any transition from a Gram negative character. The narrow zone between the membrane of the aleurone body and the internal matrix was most satisfactorily shown with Loeffler's alkaline methylene blue. This might have been due to the shrinkage of the amorphous protein elements by the alkaline solution. They were unstained against a faint blue background by the negative method of Barlow's crystal violet technique. The granules or globoid elements within these protein bodies were especially conspicuous when treated with a 1 per cent solution of aqueous erythrosin or with Ziehl's carbol fuchsin. There was no evidence of acid-fast properties. The aleurone bodies were stained a deep uniform red by Dorner's spore technique.

Flemming's triple stain afforded the best means of differentiating the aleurone from the cellular structures in tissue preparations. The cytoplasmic remnants and walls of the plant cells stained orange, the nuclear fragments dull red, and the nucleolus brilliant red, while the aleurone bodies appeared as granular, vacuolated, rose-red inclusions. Starch grains which stain a deep blue by this technique were rarely seen within the bacteroidal cells, although they were frequently observed in the innermost layers of the nodule cortex. With Heidenhain's solution the aleurone in young nodule tissues stained with the same intensity as the nucleolus, whereas in old material it possessed little affinity for the dyes and appeared homogeneously granular or vacuolated (fig. 11). Wallin and Bensley's techniques gave erratic results. In young peripheral cells near the cambial ring a majority of the aleurone bodies showed marked affinity for the acid fuchsin; in old cells of the bacteroidal area of the same nodule they stained green. In many instances both conditions were exhibited in the same cells.

LECOMTE (16) appears to have been the first to note the presence of aleurone in nodules of the peanut plant, although he credited GODFRIN (13) with priority.[1] LECOMTE regarded this substance as a rich nitrogenous reserve and assumed that it played a definite role in the symbiotic nitrogen-fixation process. To date, the presence of aleurone in nodules of other species of the Leguminosae has not been reported. Various considerations may be given to the origin and nature of the aleurone grains and their function in the root nodules of the peanut plant.

[1] LECOMTE (p. 304) inadvertently substituted in his quotation of GODFRIN's work (p. 32) the words "des tubercules" for GODFRIN's "de ce cotylédon." No attention was given by GODFRIN to the study of root nodules.

Aleurone is a common ergastic proteinaceous storage product in seeds of such plants as the castor bean, lupine, corn, etc. In different species it varies considerably in color, form, and structure. In seeds of *Pisum* the aleurone grain consists only of an amorphous substance; in others the ground substance incloses a rounded globoid particle, a crystal of calcium oxalate, or a large angular albuminous crystalloid. The best known aleurone bodies are probably those of the deeply lying endosperm cells of the castor bean, which consist of a protein ground substance, a crystalloid, and a globoid composed of a double phosphate of calcium and magnesium together with certain organic constituents.

Early investigators have shown that aleurone grains are formed in vacuole-like cavities as the seed matures. DANGEARD (8) was among those to emphasize these cavities as actual sap vacuoles, since he maintained that the vacuolar material passes through a reticular stage and breaks up into a number of small vacuoles in which the gradually condensing constituents differentiate as crystalloid, globoid, and ground mass. MOTTIER (23), however, has claimed that aleurone formation involves the activity of permanent plastid primordia, which aggregate in large numbers in the vacuole-like cavities, where their combined products unite to form the aleurone grains. Accordingly he has assumed the primordia for the aleurone bodies to be mitochondria-like, and their formation analogous to that of leucoplasts and chloroplasts from plastid primordia (14, 18, 20, 22).

The reports of these investigators are of interest in connection with the observations made in the course of this study. It seemed apparent from the initial appearance of the aleurone grains in the infected cells of the peanut nodule that there was a relationship between their formation and the presence of mitochondria. With the onset of aleurone formation there was always a decrease in the numbers of mitochondria accompanied by a clearing of the cytoplasm. In the mature cells of the bacteroidal area mitochondria were not distinguishable; whether this was due to their complete disappearance or to their being masked by the cellular contents of aleurone bodies could not be determined. The consistent occurrence of aleurone in the cytoplasm between the central vacuole and the cell wall and the absence of the many small vacuoles so conspicuous in clover and alfalfa nodules lessen the likelihood that their formation was concerned with vacuole-like cavities. The fact that aleurone was formed only in the infected cells similarly suggests a relationship with the rhizobia. It is believed that this relationship differs from that involving starch production, however, since MCCOY (19) found that starch deposits occurred primarily in the uninfected bacteroidal cells of the bean nodule.

Aleurone grains were sparse in young nodules from plants undergoing nitrogen lack, although in nodules showing approximately the same stages of development—taken from plants showing greening of the leaves—they were common. Yet a sig-

nificant difference between their numbers per cell in the mature nodules produced by the effective and the ineffective strains was not obtained. Little explanation can be offered for the presence of aleurone in nodules of the peanut plant and its apparent absence in nodules on other leguminous species. The fact that aleurone grains produced in certain species are soluble in water and in others are insoluble should not be overlooked. It is probable that the chemical complex of aleurone is affected by the physiological nature of the peanut plant, since oils occur in such large quantities. Such a condition may account for its insolubility.

Disintegration of the aleurone bodies, as evidenced by their mottling, irregularity in shape, and diminution in size, was apparent in all sections of moderately old nodules. As disintegration progressed, the bodies contained circular or angular cavities in lieu of the previous granular or crystalloid centers. Differences in the appearance of the aleurone found in mature and old nodules is noted by comparing figure 10 with figure 11. In general these results are in agreement with the observations of LECHTOVA-TRNKA (**15**) in regard to the "spherules," which she assumed to be large involution forms of rhizobia, and also with GODFRIN'S (**13**) description of aleurone dissolution in cotyledons of the peanut plant. The probability that aleurone is ultimately transformed by enzymes produced by the rhizobia is not to be excluded. Old empty nodules, the bacteroidal contents of which had undoubtedly been absorbed by the plant, were frequently found adhering to the roots. In many instances these old shells were totally devoid of cracks or crevices through which the contents might have exuded into the soil. On no occasion were hollow-center nodules found on the plants inoculated with the strains of group C. It is likely that, since the ineffective nodules are minute and since they apparently undergo complete disintegration in a relatively short time, the old forms escaped observation at the time of sampling. THORNTON (**37**) has recently commented upon the rapidity with which the ineffective nodules of red clover disintegrate.

DEGENERATION OF NODULE

One of the first indications of nodule disintegration was the production of a layer of cells with heavy suberized walls at the base of the nodule, more or less perpendicular to the main tube of the vascular strand entering from the root. This suberized cell layer encroached upon and later severed the central vascular system of the nodule. Thereafter the vascular bundles in the nodule cortex lost many of their staining properties, and the xylem and phloem elements became less distinct. This suberization (fig. 7), formed between the base of the empty nodule and the mother root, eventually became a seal on the root surface after the nodule had completely decayed. It undoubtedly functions in inhibiting penetration of many contaminating microorganisms into the wound incurred in the root tissues when the old nodule sloughs off. About the time suberization became evident, masses

of rhizobia in zoogloea-like strands appeared between the walls of the cells in the innermost bacteroidal region (fig. 11). Many of these strands extended for considerable distances through the bacteroidal zone and appeared multi-branched when traced in serial sections. The rhizobia in these strands had marked affinity for many of the stains used and were prominent in comparison with the faintly stained background of disintegrating aleurone bodies and cellular contents. According to THORNTON (**35**), the presence of bacteria in intercellular spaces is due to their attack on the cell walls as a result of an unbalanced equilibrium of the food supply brought about by the inefficiency of the vascular system. The observations in this study lend support to this explanation. In general, necrosis progressed from the innermost area of the bacteroidal zone to the periphery of the nodule, with the result that the plant absorbed the major part of the nodule contents prior to disintegration of the vascular branches and collapse of the nodule cortex. Many old nodules were sectioned wherein only faint remnants of the bacteroidal area remained near the cortical periphery (fig. 7).

Old nodules were recognized by their dark color, wrinkled surfaces, and soft texture. In the early stages of degeneration the bacteroidal areas had a mucilaginous consistency and could readily be lifted out of the cortical covering with a dissecting needle when the nodules were cut in halves. In advanced stages of decay, splits in the nodule cortex —from which there exuded a slimy liquid—were occasionally noted. This exudate consisted of protozoa, filamentous fungi, large bacilli, and an abundance of short, rod-shaped forms typical of rhizobia. It is undoubtedly in this manner that the root nodule bacteria return to the soil.

RELATIONSHIP OF NODULES TO ROOTS

Nodules of the peanut plant and lateral roots have the following similarities in structure and development: (*a*) both arise in the same layer of cells, the pericycle; (*b*) both points of origin are in the cells adjacent and in juxtaposition to protoxylem points; (*c*) both structures digest or push their way through the root cortex; (*d*) during their existence the nodules, as well as the lateral roots, are entirely free of the cortex of the mother root; and (*e*) in regard to the vascular system the strand supplying the nodule and the lateral root can be traced to only one primary xylem group.

Despite these similarities, it is not to be understood that peanut nodules are modified lateral roots. These data, as well as those presented by others, emphasize the existence of nodules as separate and distinct entities from root structure, since they (*a*) arise only as a result of bacterial infection, (*b*) contain a definite bacteroidal area within a distinct cortex, and (*c*) possess no central stele, no area analogous to a root cap, and no epidermis.

Summary

1. Each of fifty-nine strains of rhizobia, isolated from a variety of leguminous plants, proved infective upon the roots of the peanut plant. Seventeen strains markedly enhanced plant growth and nine were effective to a less extent, in contrast to thirty-three strains which were decidedly non-beneficial.

2. All nodules, irrespective of size and the sources of inocula, were located in root axils. Those produced by the non-beneficial strains were inconspicuous and tended to be visible only when the roots were immersed in water.

3. Abnormal nodose formations, which proved to be merely hypertrophied parenchymatous tissue containing heavy deposits of starch, were frequently found at the base of the rootlets of plants deficient in nitrogen.

4. Infection of roots results from invasion of rhizobia through the ruptured tissue at the site of lateral root emergence. Lateral or normal root hair formation is rare, whereas tufted whorled rosettes of hairs are common in the root axils. No data were obtained supporting root hairs as avenues of infection.

5. Origin of the nodule is in the pericyclic cells adjacent and in juxtaposition to the protoxylem strand from which a rootlet had emerged. Until such emergence the nodule remains within the endodermis. Dissemination of rhizobia throughout the bacteroidal area is effected by passive transmission of rhizobia to new cells during the laying down of the cell plate after mitosis.

6. Differentiation of the nodule results from the formation of a peripheral meristem which serves in the production of a nodule cortex and a bacteroidal area. A branched vascular system supplying the nodule surrounds the bacteroidal area and connects with the xylem and phloem elements of the main root through a single connecting strand at the base of the nodule.

7. Spherical plastid-like bodies, identified as aleurone grains, were abundant in the infected cells of the bacteroidal area. It is thought that aleurone is directly concerned with the bacteria-plant symbiosis, and is of significance in the nitrogen-fixation process.

8. Initial stages in degeneration of the nodules are characterized by the formation of a suberized layer of cells at the base of the nodule, thereby cutting off the vascular supply. In consequence the bacteroidal area assumed a slimy consistency and rhizobia invaded the intercellular spaces. The bacteroidal area of many nodules was found to be absorbed by the plant prior to collapse of the old nodule cortex.

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CHROMOSOME CATENATION IN OENOTHERA

K. T. JACOB

(WITH TWENTY-FIVE FIGURES)

Introduction

The cytology of *Oenothera* is of special importance, since it was in this genus that various correlations between the chromosome content or behavior and genetic phenomena were first described. The first tetraploid mutant (*O. gigas*) was investigated by GATES (16), who suggested that tetraploidy here arose through failure of the chromosomes to separate in the first or early division of the fertilized egg. It was in *O. rubrinervis* that the first instance of nondisjunction was reported (15) and the first instance of triploidy dealt with (17). The first trisomic investigated cytologically (18) also belongs to this genus. Parallel mutations were described in this genus in 1912, and their importance in evolution was pointed out later (19).

GATES (20) listed the chromosome numbers in *Oenothera*, and GATES and FORD (27) recorded the catenations so far investigated. By catenation is meant the linkage of the chromosomes end to end in the prophase of meiosis to form one or more rings, and the opinion that the particular arrangement is fixed for each species or type is now generally held. The maximum catenation is usually the significant one, although derivative associations are observed with variable frequency, probably due to failure of chiasma formation between the pairing bivalents (4). It is well known that in *Oenothera* species with chromosome catenation there is also genetic linkage, so that only two types (Renner complexes) of male and female gametes are generally produced. Also each chromosome in the ring consists of two ends and a central portion, these central portions being different while the two ends of each chromosome correspond respectively with one end of two other chromosomes (7).

The present paper embodies an investigation of the catenations of four species and ten hybrids, three of the hybrids including two types each. The plants were grown by Professor R. R. GATES at the Courtauld Laboratory in Regent's Park, London. To him my thanks are due for kindly supplying the material for this investigation. These are all species, chiefly from eastern Canada, described in a monograph (22), but the first four listed and the male parent in no. 10 are undescribed forms named after their place of origin. The plants are listed in table 1.

Cytological technique

The cytological material of these species and the hybrids was selected by examining one anther of each bud in a drop of Belling's acetocarmine. The buds were

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2. All nodules, irrespective of size and the sources of inocula, were located in root axils. Those produced by the non-beneficial strains were inconspicuous and tended to be visible only when the roots were immersed in water.

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CHROMOSOME CATENATION IN OENOTHERA

K. T. JACOB

(WITH TWENTY-FIVE FIGURES)

Introduction

The cytology of *Oenothera* is of special importance, since it was in this genus that various correlations between the chromosome content or behavior and genetic phenomena were first described. The first tetraploid mutant (*O. gigas*) was investigated by GATES (**16**), who suggested that tetraploidy here arose through failure of the chromosomes to separate in the first or early division of the fertilized egg. It was in *O. rubrinervis* that the first instance of nondisjunction was reported (**15**) and the first instance of triploidy dealt with (**17**). The first trisomic investigated cytologically (**18**) also belongs to this genus. Parallel mutations were described in this genus in 1912, and their importance in evolution was pointed out later (**19**).

GATES (**20**) listed the chromosome numbers in *Oenothera*, and GATES and FORD (**27**) recorded the catenations so far investigated. By catenation is meant the linkage of the chromosomes end to end in the prophase of meiosis to form one or more rings, and the opinion that the particular arrangement is fixed for each species or type is now generally held. The maximum catenation is usually the significant one, although derivative associations are observed with variable frequency, probably due to failure of chiasma formation between the pairing bivalents (**4**). It is well known that in *Oenothera* species with chromosome catenation there is also genetic linkage, so that only two types (Renner complexes) of male and female gametes are generally produced. Also each chromosome in the ring consists of two ends and a central portion, these central portions being different while the two ends of each chromosome correspond respectively with one end of two other chromosomes (**7**).

The present paper embodies an investigation of the catenations of four species and ten hybrids, three of the hybrids including two types each. The plants were grown by Professor R. R. GATES at the Courtauld Laboratory in Regent's Park, London. To him my thanks are due for kindly supplying the material for this investigation. These are all species, chiefly from eastern Canada, described in a monograph (**22**), but the first four listed and the male parent in no. 10 are undescribed forms named after their place of origin. The plants are listed in table 1.

Cytological technique

The cytological material of these species and the hybrids was selected by examining one anther of each bud in a drop of Belling's acetocarmine. The buds were

collected on bright days between 10:30 and 12:00 A.M., and stripped of their sepals and petals, the tops of the anthers being cut off with a sharp razor. They were then dipped in Carnoy's fluid (modified by Semmens) for 1–2 seconds, rapidly rinsed in water, and fixed in Flemming's medium solution. An air pump was used only in those cases where the buds failed to sink, but it was usually unnecessary. Navashin's was also used as a fixative, but not with very satisfactory results.

The fixed material was washed for 12–24 hours in running water and dehydrated by the chloroform method. Sections were cut 14–18 μ thick. Material fixed in Flemming was bleached before staining in a mixture of 20 volumes of H_2O_2 and 60 volumes of 80 per cent alcohol by keeping it for 4–5 hours near a 60 watt lamp. Chlorine water as well as nascent chlorine was tried for bleaching, but the results were not entirely satisfactory. The slides were stained by the gentian violet-iodine-chromic acid technique. The Feulgen fast green method (**45**) did not prove satisfactory. These staining methods have all been described elsewhere (**32**).

The laborious nature of the section method just described impelled the use of a rapid and reliable smear technique (**6**) in those cultures where the buds were of medium size. By this method about 30–40 slides could be made in a day, but a considerable number had to be discarded owing to desiccation of the pollen mother cells on the slides before fixing. The buds were selected by examining one anther of each in Belling's acetocarmine. Although the chromosomes could not be seen, owing to the granular nature of the cytoplasm, the stage of development of the pollen mother cells could be ascertained by this pre-examination. The ordinary smear method with the scalpel or slide was unsatisfactory, since the anthers were slippery and the juices from the anther wall tissue interfered with fixation and prevented most of the pollen mother cells from adhering to the slide. Best results were obtained by holding the selected bud base downward, after removing the sepals and petals, and cutting off the tops of the anthers with a sharp scalpel. The buds were then squeezed, holding them upright, and the emerging pollen mother cells smeared on to the slide. The slides were immediately plunged into the fixing fluid, prepared as follows:

Solution A:	
Chromic acid	5 gm.
Glacial acetic acid	50 cc.
Distilled water	320
Solution B:	
Formalin	100 cc.
Distilled water	275

Equal volumes of solutions A and B were mixed just before use. Fixation took 4–5 hours. The slides were then kept in solution A for about 10 minutes, washed, and stained by the gentian violet-iodine-chromic acid technique.

Observations

Stages prior to diakinesis were not studied. Table 1 lists the catenations observed.

An attempt was made to fix the relative positions of the nucleolar chromosomes in the ring, but it was not possible in all the plants. The importance of nucleolar

TABLE 1

LIST OF CATENATIONS

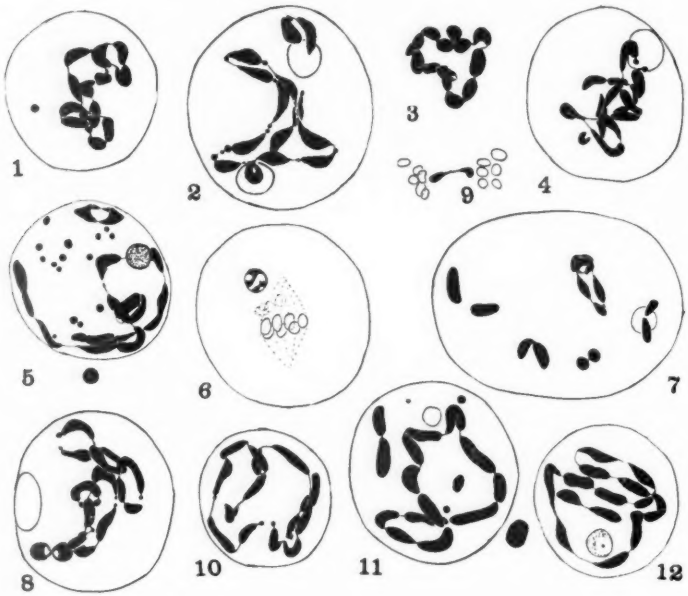
CULTURE NO.*	PLANT	MAXIMUM CATENATION OBSERVED
	Species:	
1	O. St. Eustache (Quebec)	⊙ 14
12	O. Ile aux Coudres (Quebec)	⊙ 14
13	O. Burnham (Somerset, England)	⊙ 10, ⊙ 4
18	O. Fort Coulonge (Quebec)	⊙ 14
	Hybrids (F_1):	
148	O. angustissima Gates×O. angustissima quebecensis Gates	⊙ 8, ⊙ 4, 1_{II}
149	O. angustissima quebecensis×O. angustissima	⊙ 8, 3_{II}
150	O. hazelae Gates×O. hazelae var. parviflora Gates	⊙ 10, 2_{II}
152	O. biformiflora cruciata Gates×O. angustissima quebecensis Gates	⊙ 4, 5_{II}
154	O. biformiflora cruciata×O. rubricapitata Gates	
	Type I: flowers broad-petalled	⊙ 10, 2_{II}
	Type II: flowers narrow-petalled	⊙ 12, 1_{II}
155	O. eriensis Gates×O. Long Island (species II)	⊙ 12, 1_{II}
157	O. repandodentata Gates×O. eriensis Gates	⊙ 10, ⊙ 4
159	O. albinervis Gates (from Fargo, N.D.)×O. rubricapitata	⊙ 12, 1_{II}
160	O. albinervis Gates (from Barrie, N.D.)×O. rubricapitata	
	Type I: without red papillae	.....
	Type II: with red papillae	⊙ 8, 3_{II}
161	O. rubricapitata Gates×O. albinervis Gates	
	Type I: without red papillae	⊙ 10, ⊙ 4
	Type II: with red papillae	⊙ 14

* No. under which the plants were grown in the summer of 1938.

studies was first emphasized by HEITZ (**30, 31**). Earlier, DE MOL (**37**) found that primary diploids have two nucleoli; triploids, three; and tetraploids, four. Other investigators have subsequently dealt with this problem in other plants, and GATES (**25, 26**) reviewed the relevant literature.

Figure 4 shows the two satellited chromosomes attached to the nucleolus in the hybrid *O. angustissima*×*O. angustissima quebecensis*. The maximum catenation observed was ⊙8+⊙4+1_{II} (table 1), but in the figure the ring of 8 is broken up into a chain of 7 and 1 univalent. If the positions of the chromosomes in the rings are fixed (**24**), it is safe to assume that the ring of 8 in this case includes the two satellited chromosomes. Figure 5 shows the nucleolus in association with the chain

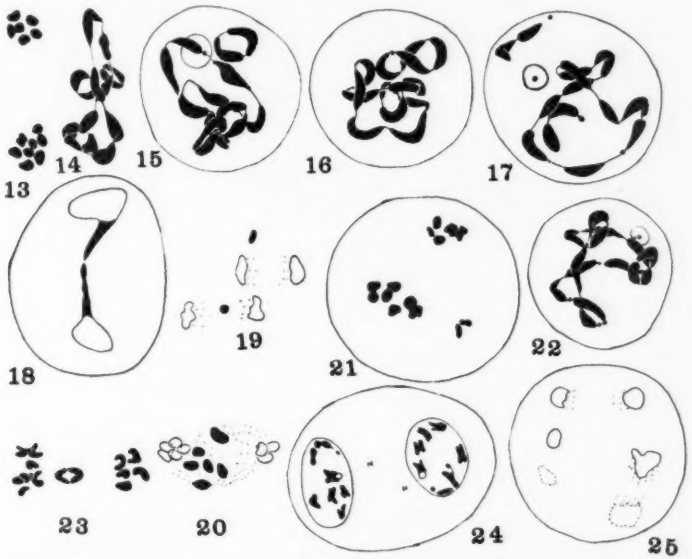
of 8 in the reciprocal hybrid *O. angustissima quebecensis*×*O. angustissima*, where the maximum catenation observed was $\odot 8+3_{II}$. Here also the nucleolar chromosomes are included in the $\odot 8$. Figure 7 shows that the nucleolar chromosomes



FIGS. 1–12.—Fig. 1, *O. St. Eustache*, diakinesis showing ring of 14. Fig. 2, *O. Burnham*, same with ring of 10, 2 bivalents, and a number of tandem terminal granules. (Maximum catenation is ring of 10+ring of 4. Two nucleoli seen, one on ring of 10 and other on ring of 4.) Fig. 3, *O. Fort Coulonge*, late diakinesis showing ring of 14. Fig. 4, *O. angustissima*×*O. angustissima quebecensis*, 2 satellited chromosomes attached to nucleolus at diakinesis (maximum is ring of 8+ring of 4+1 bivalent). Here satellited chromosomes are included in ring of 8, which is broken up. Fig. 5, *O. angustissima quebecensis*×*O. angustissima*, diakinesis showing chain of $8+3_{II}$. Number of bodies seen inside nuclear membrane and one outside. Fig. 6, same hybrid, micronucleus at metaphase II, formed from single chromosome. Fig. 7, *O. biformiflora cruciata*×*O. angustissima quebecensis*, diakinesis showing ring of $4+5_{II}$; one bivalent attached to nucleolus. Fig. 8, same hybrid, diakinesis showing 16 chromosomes, terminal granules, and interlocking of bivalents. Fig. 9, same, anaphase I showing lagging due to interstitial chiasma. Fig. 10, *O. biformiflora cruciata*×*O. rubricapitata*, type I. Diakinesis showing ring of $10+2_{II}$; terminal granules seen. Fig. 11, type II. Diakinesis showing ring of $12+1_{II}$. Four bodies inside nuclear membrane and one outside. Fig. 12, *O. eriensis*×*O. Long Island* (species II), diakinesis showing ring of $12+1_{II}$.

form a free bivalent in the hybrid *O. biformiflora cruciata*×*O. angustissima quebecensis*, where the nucleolus is found in association with this bivalent. Similarly, in culture no. 160 (table 1), in type II with red papillae, the nucleolar chromosomes are included in the $\odot 8$ (fig. 15); and in culture no. 161, type I, they are on

the ring of 10 while in type II they are naturally in the ring of 14, since all the chromosomes are linked together (fig. 22). In the *O. Burnham* strain two nucleoli are produced, one attached to the ring of 10 and the other to the ring of 4 (fig. 2). In this case the ring of 4 is broken up into two rod bivalents.



FIGS. 13–25.—Fig. 13, *O. eriensis*×*O. Long Island* (species II), metaphase II showing 8 chromosomes at one plate and 6 at other. Fig. 14, *O. albinervis*×*O. rubricapitata*, type II. Diakinesis showing ring of $8+3_{II}$. Fig. 15, type II. Diakinesis showing chain of $8+3_{II}$; one satellite in chain of 8. Figs. 16–21, *O. rubricapitata*×*O. albinervis*, type I. Fig. 16, diakinesis showing ring of 10+ring of 4. Fig. 17, showing ring of $10+2_{II}$ and terminal granules. Fig. 18, anaphase I showing bridge. Fig. 19, anaphase II showing 2 lagging chromosomes. Fig. 20, anaphase I showing 6 lagging chromosomes. Fig. 21, metaphase II showing 7 chromosomes at one plate and 5 at other; two exhibit noncongression. Figs. 22–25, *O. rubricapitata*×*O. albinervis*, type II. Fig. 22, diakinesis showing ring of 14 and terminal granules. Fig. 23, anaphase I showing one lagging ring bivalent. Fig. 24, interkinesis showing 2 nucleoli, one at either pole; satellited chromosomes not attached to nucleolus. Fig. 25, anaphase II showing 6 poles.

Varying numbers of free bodies were found inside the nuclear membrane and in some cases in the cytoplasm too, the significance of which is not clear. Figure 5 shows 15 such bodies inside the nucleus and one outside it. These bodies stain with gentian violet in the same way as do the chromosomes.

Varying numbers of granules were found on the threads connecting the ends of the chromosomes at diakinesis. The noncommittal name, terminal granules, suggested by GATES, has been used for them. Since such granules are not present in the somatic chromosomes it is perhaps safe to assume that they may have been

brought about as a result of pairing at pachytene. CATCHESIDE (4) has figured such granules and assumed that they were caused by incomplete terminalization of chiasmata. This explanation is plausible in cases where there is only a single granule between the chromosomes. Sometimes two or three tandem granules (fig. 2) are seen, however, the origin of which is not clear. They may have been caused by the despiralization of the ends of chromosomes, with the result that at diakinesis, when the chromosomes repel each other, the despiralized portions may be relatively more drawn out.

Interlocking of bivalents was often seen. Figure 8 shows one ring bivalent interlocked with a ring of four in *O. biformiflora cruciata*×*O. angustissima quebecensis*. The same figure shows another bivalent interlocked with a chain of three on one side and one bivalent on the other. The occurrence of interlocking is due to distribution of the threads at zygotene. If two chromosomes pair in such a way that a third chromosome of another pair remains between the pairing threads, chiasma formation at both ends in each pair will bring about interlocking of the bivalents. In *Oenothera* this is a common phenomenon, but it has been observed in other organisms like *Dendrocoelum* (29), *Viviparus* (1), *Allium* (35), and rice (41).

Figure 8 shows 16 chromosomes instead of the usual 14 in *O. biformiflora cruciata*×*O. angustissima quebecensis* at diakinesis. This higher number may have arisen by the separation of certain daughter chromosomes immediately after their formation by the prophase split during pre-meiotic mitosis, as suggested by GATES (18) in somatic mitosis of *O. lata*, where he observed cells showing 12, 16, 20, or 21 chromosomes instead of the usual 15. Or it may have arisen owing to double nondisjunction at anaphase of the pre-meiotic mitosis, with the result that both halves of two chromosomes pass to the same pole. If the latter contention should be valid, one naturally expects another pollen mother cell with a lesser number of chromosomes near by. Such a cell was not observed, but this may have been due to the fact that it had been lost during smearing, or it may have lagged in the further developmental stages and got eliminated.

On the assumption that each chromosome consists of three parts (two distal pairing segments homologous with the pairing segments of the adjacent chromosomes in the ring and one median differential region) and that the aggregate of the differential regions accounts for genetic differences between the two gametic complexes, the zigzag arrangement of the ring or chain on the spindle is the usual configuration that will give rise to viable gametes. CATCHESIDE (7), however, points out that there is as yet no evidence in support of the hypothesis that every chromosome has such a differential region.

On the other hand, the failure of this regular zigzag arrangement may lead to the formation of two daughter nuclei with different numbers, one having an extra chromosome and the other lacking one. The result of such nondisjunction is

easily discernible at metaphase II. Figure 13 shows the polar view of metaphase II, with 8 chromosomes at one plate and 6 at the other in *O. eriensis*×*O. Long Island* (species II). Other variations found at this stage are 8 and 5, making a total of 13; and 9 and 6, making a total of 15 in *O. rubricapitata*×*O. albinervis*, in plants devoid of red papillae. This indicates nondisjunction in the last pre-meiotic mitosis. The first recorded observations on nondisjunction were made by GATES (**15**) in the pollen mother cells of *O. rubrinervis* and by DAVIS (**11, 12**) in *O. biennis* and *O. lamarckiana;* the significance of these observations in relation to the discovery that *O. lamarckiana* mut. *lata* had 15 somatic chromosomes was soon recognized. FORD (**14**), in an analysis of nondisjunction in *Oenothera*, concluded that 49 different 8-chromosome gametic types were possible from a ring of 14 chromosomes, on the assumption that the presence of at least one representative of each pairing segment is a necessary condition of gametic viability. He also suggested that nondisjunction increases with increasing number of chromosomes in the ring.

Lagging chromosomes were often observed at anaphase I in nearly all the plants. Figure 23 shows a ring bivalent lagging at this stage in *O. rubricapitata*×*O. albinervis*, plants with red papillae, where the maximum catenation is a ring of 14. Figure 20 shows 6 chromosomes lagging. The spindle in this case is distorted, and such cells may fall behind in the further developmental stages and may be totally eliminated. Figure 9 shows a lagging bivalent, probably due to interstitial chiasma, in *O. biformiflora cruciata*×*O. angustissima quebecensis*. This failure of complete terminalization of the chiasma may be due to arrest because of the change in homology. Similar conditions have been observed before (**28**) in *O. lata*.

Another interesting feature in *O. rubricapitata*×*O. albinervis* at anaphase I is the presence of a dicentric chromatid which connects the two poles to form a bridge. This may be the consequence of pairing between inverted segments, followed by crossing-over in the relatively inverted regions. The conditions necessitating such pairing have been dealt with (**32**), so that it is not necessary to cite the various possible configurations here. At pachytene, in favorable material like *Zea* (**36**), *Chorthippus* and *Stauroderus* (**10**), and *Tulipa* (**47**), inversion loops were actually observed, but this was not possible in the present material. At metaphase I the inversions are not usually recognizable or inferred, except when the individual chromosomes remain distinct, as in *Paeonia* (**8**), *Phalaris* (**42**), etc. At anaphase I there is no doubt about the occurrence of crossing-over in the relatively inverted segments giving rise to a dicentric chromatid, as just mentioned. This dicentric chromatid stretches across the two poles, forming a bridge (fig. 18). In the figure the bridge has just broken, owing to tension. A dicentric chromosome may also arise through reciprocal translocation between two chromosomes. The fragments left may not be easily visible if they are too small.

Figure 24 shows a pollen mother cell at interkinesis in *O. rubricapitata*×*O. albinervis*. Here two nucleoli are seen, one at each pole, with a chromosome attached to each. The nucleolus at each pole appears to have been organized in connection with the chromosome to which it is seen attached at this stage. The chromosomes show also their dual nature. It is surprising to find that the apparently satellited chromosomes (two at one pole and one at the other) have not organized nucleoli at all. No definite conclusions can be drawn from such few observations, but it has been established in other plants (**30, 31**) that nucleoli arise usually in connection with the satellited or secondarily constricted chromosomes. It is not clear whether this rule will hold in the case of a complex heterozygote such as *Oenothera*. This problem could not be investigated further, as the material was not treated for such a study. LATTER (**34**), however, did not observe nucleoli at interkinesis in *Lathyrus*. BHATIA (**2**) did find nucleoli arising at this stage in wheat.

Figure 21 shows a polar view of metaphase II with 7 chromosomes at one pole and 5 at the other. Two are lost in the cytoplasm. Such chromosomes may develop their own spindles and divide, as will be shown later.

Figure 6 shows a micronucleus at metaphase II in *O. angustissima quebecensis*× *O. angustissima*. Here one plate is seen in side view showing 7 chromosomes, and at a lower focus the polar view of the other metaphase plate shows 6 chromosomes; so it is evident that the micronucleus is formed from a single chromosome. It is not clear at what stage this aberration took place. Probably this micronucleus may have been formed by a single lagging chromosome at anaphase I, which failed to get included in the interkinesis nuclei. If this is correct, then it is not clear why the nuclear membrane did not disappear along with the membranes of the interkinesis nuclei. The chromosome in the micronucleus is still in a resting condition.

Lagging chromosomes were also found frequently at anaphase II in most of the plants (fig. 19). Figure 25 shows the chromosomes grouped at six poles instead of at four in *O. rubricapitata*×*O. albinervis*, plants with red papillae. The extra poles may have been formed by some of the lagging chromosomes developing their own spindle, or it may represent the further developmental stages of a tripolar spindle at anaphase I. Tripolar spindles have been observed in other plants, like rice, in both the first and second divisions (**33, 44, 41**).

Discussion

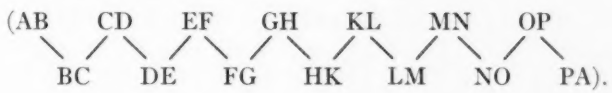
Among the many structural changes characteristic of species evolution, segmental interchange occupies a prominent position, as it has not only a survival value but under certain conditions is specially favored by nature also. Many of the mutations in *Oenothera* depend upon the structural complexity of the chromosomes, which must have arisen from the noncomplex condition through translocations. GATES (**24**) regards the origin of the linked condition of the chromosomes in

Oenothera as due to crossing between different species or types in which one or more pairs of chromosomes are nonhomologous, this condition having arisen by crossing-over or exchange of ends between nonhomologous chromosomes in a species. According to him (**21**) the ancestral forms of *Oenothera* were homozygous with large flowers, while with decrease in flower size brought about by mutations there was an increase in the catenation of the chromosomes. In *Oenothera* this interchange is maintained in a heterozygous state by a special lethal mechanism associated with homozygosity, while in some other ring-forming plants, like *Rhoeo*, *Campanula*, etc., such mechanisms are only in the process of evolution, as evidenced by the arrangement of the rings.

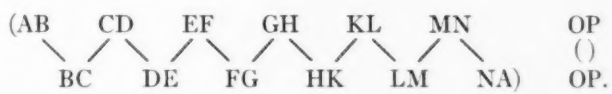
Thus on the assumption that the linked condition of the chromosomes in *Oenothera* arose as a result of successive interchanges in the ancestry of the plant, a plant with the constitution

AB	CD	EF	GH	KL	MN	OP
( )	( )	( )	( )	( )	( )	( )
AB	CD	EF	GH	KL	MN	OP

will by a series of interchanges produce an individual which has a ring of 14 chromosomes having the constitution



On the other hand, segmental interchange between two chromosomes of the same complex in such a plant, like NO and PA, will produce two new chromosomes, NA and OP, so that the resulting plant will have a ring of 12 chromosomes and one bivalent with the constitution



This was inferred from the presence of a lagging ring bivalent (fig. 23) at anaphase I in *O. rubricapitata*×*O. albinervis*, which normally produces a ring of 14 chromosomes in the type with red papillae.

Segmental interchange may take place between homologous or nonhomologous chromosomes. Between homologous chromosomes it is the result of pairing of homologous threads followed by breaks and reunion of the parental threads. But between nonhomologous chromosomes segmental interchange must take place in other ways. According to MORGAN *et al.* (**38**), translocations may be caused by the

interlocking of nonhomologous chromosomes during the process of synapsis, and CATCHESIDE (5) is of the opinion that this phenomenon may be responsible for segmental interchange. Fragmentation followed by reunion of fragments with broken ends of chromosomes brings about translocation. Apparently all effective translocations are reciprocal where segments are mutually exchanged between two nonhomologous chromosomes (5). Supposed simple translocations (3) have been shown to be reciprocal where one of the segments was very small.

In regard to the mechanism of reciprocal translocation, STADLER (46) considers that it involves random breakage with subsequent reattachment of the broken ends, there being an unlimited interval between the two events. But this is hardly likely, as acentric fragments tend to get lost during mitosis. The most likely view is that of CATCHESIDE (6), who considers that the broken ends join immediately at random when the break occurs at the overlap of two chromosome threads.

DARLINGTON (9) suggests another possibility for the occurrence of segmental interchange between nonhomologous chromosomes. In an organism subject to structural changes in the process of evolution there is the probability of the translocation of an interstitial segment from one chromosome to a corresponding part of one not homologous with it. The steps leading to this involve a series of interchanges. Thus in a chromosome differentiated into three segments, MON, if M and N get interchanged with different segments from two different chromosomes, a new chromosome, POR, may result, leading to the re-duplication of the O segment; and pairing of these two different chromosomes will be the basis for a new interchange—MR and NP chromosomes. In *Pisum*, in a ring of 6 chromosomes (43) crossing-over in the homologous interstitial segment in an otherwise nonhomologous chromosome (as just shown) was the cause of a new segmental interchange.

Segmental interchange may be induced by the action of X rays, when its frequency is greatly increased, as in *Drosophila* (13, 39), *Zea mays* (46), etc. NAVASHIN and GERASSIMOVA (40) found this phenomenon in seeds germinated after a long period of dormancy. Segmental interchange has been inferred as a result of hybridization in geographical races, as in *Pisum* and *Datura*.

Although segmental interchange between nonhomologous chromosomes occurs in plants like *Pisum* and *Datura*, and a chromosome ring arises as a result of hybridization between the different races involving the interchange, the heterozygous state will not be advantageous to the plant since it produces sterility, owing to the random orientation of the ring on the spindle. In the artificially produced interchange heterozygotes in *Zea*, *Nicotiana*, etc., the same condition results and no further evidence is found of the evolutionary stages leading to the highly evolved complex heterozygote condition as in *Oenothera*.

Summary

1. Chromosome catenations in *Oenothera* were investigated. The positions of the nucleolar chromosomes in the rings were ascertained in some cases, on the assumption that the particular arrangement is fixed for each plant. In *O. angustissima* ×*O. angustissima quebecensis* a pair of satellited chromosomes, which are usually included in the ring of eight, were found attached to the nucleolus at diakinesis.

2. "Terminal granules" were found at either end of the chromosomes at diakinesis, sometimes up to three between two linked chromosomes. When only a single granule was present, apparently it was brought about by failure of the terminalization of chiasmata. The tandem condition of the granules may be due to despiralization of the chromosomes at portions near their ends, enhanced by the repulsion between the chromosomes at diakinesis.

3. A dicentric chromatid was observed connecting the two poles at anaphase I in *O. rubricapitata*×*O. albinervis*. Lagging chromosomes were often found at anaphases I and II. Micronuclei were observed in a few pollen mother cells at metaphase II in *O. angustissima quebecensis*×*O. angustissima*, which involved only a single chromosome in one case. They may have been caused by a lagging chromosome at anaphase I or at the anaphase of the pre-meiotic mitosis.

4. In one pollen mother cell at anaphase II in *O. rubricapitata*×*O. albinervis*, plants with red papillae, six poles were observed instead of the usual four. The extra poles may have been due to the lagging chromosomes at first division developing their own spindle, or they may represent the further developmental stages of a tripolar spindle at first division.

5. A lagging ring bivalent was observed at anaphase I in *O. rubricapitata*×*O. albinervis* in the type with red papillae which normally produces a ring of 14 chromosomes. This may be due to segmental interchange between two chromosomes of the same complex. Conditions facilitating segmental interchange are discussed.

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EFFECTS OF VITAMIN B_1 UPON THE DEVELOPMENT OF SOME FLOWERING PLANTS

CHARLES L. HAMNER

(WITH FOUR FIGURES)

Introduction

Within the last several years there has been great interest in the possible effects of vitamin B_1 on the growth and development of green plants of economic importance. Although not yet extensive, there is an increasing amount of literature in technical journals (**1, 2, 3**) and in various publications devoted to horticultural interests. It has been stated that very large increases in the amount of dry weight accumulated occur when small quantities of vitamin B_1 are added to the nutrient medium in which the plants are grown. Such increases are reported as being especially large when the nutrient medium is relatively low in nitrogen content or when short-day plants are grown on short photoperiod as compared with long photoperiod. More briefly it has been stated that vitamin B_1 is effective in promoting increased growth whenever plants are grown under various conditions which are presumed to be suboptimal.

Material and methods

In the work here reported, some experiments similar to those already published have been performed, and others added. Most of the work was done from March 15 to July 15, 1940, in the greenhouses at the United States Horticultural Station at Beltsville, Maryland, and at the University of Chicago, some of these being repetitions of similar experiments conducted during the previous autumn and winter, with similar end results. Among a number of plants grown, the experiments dealing only with cabbage, cocklebur, cosmos, dahlia, mustard, radish, and zinnia are reported, because similar results were obtained with all varieties of plants used.

The specific treatments are given in detail under the several experiments. For most of the cultures either white quartz sand or washed yellow river sand was used. In all cases a base nutrient composed of 0.0060 mol $Ca(NO_3)_2$, 0.0045 mol $MgSO_4$, and 0.0045 mol KH_2PO_4, together with a trace of the minor elements, was supplied at least once each week; in many cases it was applied every alternate day. The reaction of the solution was pH 4.8. An amount sufficient thoroughly to saturate the sand in any given container was used at each application. Occasionally the sand was flushed with distilled water. If the plants were grown in soil, tap water was used instead of nutrient solution.

The vitamin B_1 solution was prepared immediately before use, and never stood for more than 2 hours before application. The crystalline preparation made by

Merck and Company was used. After dissolving the specified quantity required for use at any given time in distilled water, the solution was diluted to a concentration of 0.01 mg. in 1 liter of nutrient solution. In the case of the radishes grown in soil, the B_1 was diluted with tap water. Unless otherwise noted, this concentration was employed in all the experiments. At each application of this solution enough was used to saturate the sand in each pot to the degree that a slight amount dripped from the drainage opening. All experiments were set up in such a manner that a statistical analysis of the results could be made. The general plan was to use at least 100 plants in each experiment. They were divided into groups for purposes of randomization. At least eight groups subjected to each type of treatment were used. Many of the plants were grown in small glazed crocks with side delivery openings. All the others, except the radishes, were grown in new porous clay pots which were dipped in a hot paraffin-vaseline mixture to waterproof them before being filled with sand. The containers were placed on slatted frames, raised above the level of the clean bottom of a greenhouse bench, so that none of the drainage water from any container could seep into any other. Similarly, the pots were placed far enough apart that the likelihood of solutions spattering from one to another was eliminated.

Throughout the experiments the weather was somewhat variable, with sunny and cloudy days interspersed. When possible, the greenhouse temperature was maintained at approximately 70° F., but on a few days the outside temperatures ranged somewhat higher, although never in excess of 85° F.

To maintain the conditions of short photoperiod, a framework was constructed over the greenhouse benches, and over this blankets of black sateen cloth were spread to darken the plants for any desired period. The maximum light intensity under such a blanket did not exceed one-half of a foot-candle at any time.

Every effort was made to do the work of harvesting the plants as rapidly as possible. The containers in which the plants were grown were plunged in water, the plants removed with great care, the roots washed in fresh water and then dipped in salt water to aid in removal of the sand. The plants were then blotted, divided into root and top, each sample weighed individually and immediately dried in an electric oven at 80° C. in a rapidly moving current of air. When brittle dry, the plants were weighed again. After drying and weighing, all sand still adhering to the root samples was carefully removed and weighed, and this weight subtracted from the original wet and dry weights, only the final net weights being recorded in the table.

Experimental results

Cabbage

Experiment I.—On March 30, 3000 Early Jersey Wakefield cabbage seeds were planted in white quartz sand and watered with nutrient solution. On April 9,

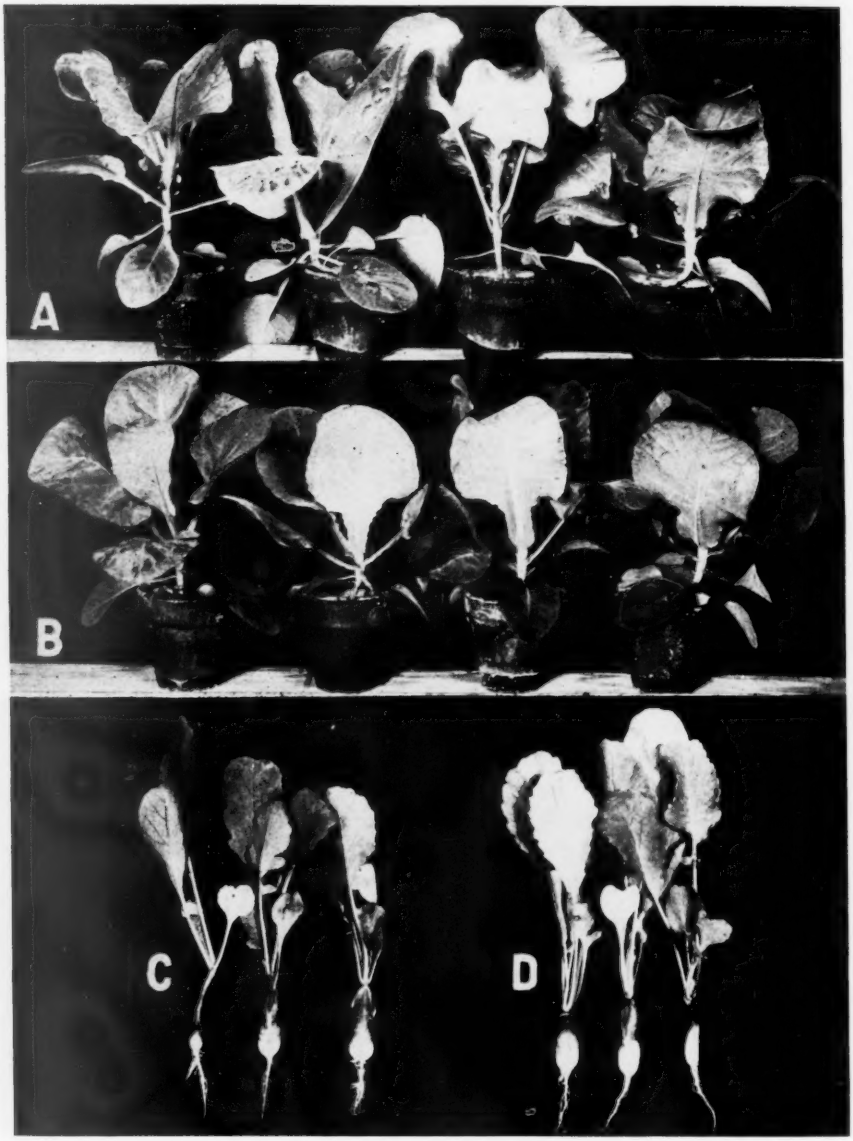

FIG. 1.—Early Jersey Wakefield cabbage (experiment I): *A*, control; *B*, with vitamin B_1 added; *C*, French Breakfast radish (experiment VIII), control; *D*, same with vitamin B_1 added.

seedlings were selected for uniformity and transplanted individually to 4-inch clay pots and placed on a greenhouse bench. All the plants received nutrient solution every day, and in addition half of them received vitamin B_1 once a week. The plants were grown in rows of eight and the treatments were randomized upon the bench. The plants grew very vigorously and were harvested on May 18. No significant differences between those to which B_1 was added and those to which it was not were observed (fig. 1*A*, *B;* table 4).

Cocklebur

Two experiments were carried out, using seeds which had been collected in the autumn of 1939 and stored out of doors during winter. Experiment II was conducted at Beltsville, Md. On March 30, 1200 *Xanthium* seeds were planted in quartz sand. After germination, a number of plants were selected for uniformity and then transplanted. Five plants per crock were used, with a total of thirty-eight crocks. All the plants were placed under a long photoperiod consisting of 17 hours of natural daylight supplemented by 100-watt Mazda lamps out of each 24-hour period. The crocks were supplied with the complete nutrient solution, and half received in addition the vitamin B_1 once each week. Growth was vigorous, although somewhat uneven, and the plants were harvested 47 days after the treatment began (table 4).

Another lot of cockleburs was grown in the greenhouses at the University of Chicago. In this experiment (III) the seeds were planted May 5. Four hundred and ninety plants were grown singly in $1\frac{1}{2}$-liter glazed crocks filled with white quartz sand and watered with nutrient solution. Until June 3 they were maintained under a long photoperiod of 17 hours of natural daylight supplemented by 200-watt Mazda lamps out of each 24-hour period. On May 15 and subsequently, half of the plants were supplied with the B_1 solution twice each week in addition to the nutrient solution. The plants given the two treatments were completely randomized on the benches. On June 3, the photoperiod for half the plants was reduced to 8 hours of natural daylight out of each 24-hour period; the others were continued as previously. The plants were harvested on June 18.

In neither experiment II nor III were significant differences obtained with respect to the vitamin B_1 treatment.

Cosmos

Several experiments were conducted. For experiment IV the variety Early Single was used. Under any conditions of culture the plants of this variety show very wide variation in rate of growth, time of blooming, size and color of flowers, and various other characters. Approximately 4000 seeds were planted in quartz sand on March 20 and placed under conditions of long photoperiod, 18 hours

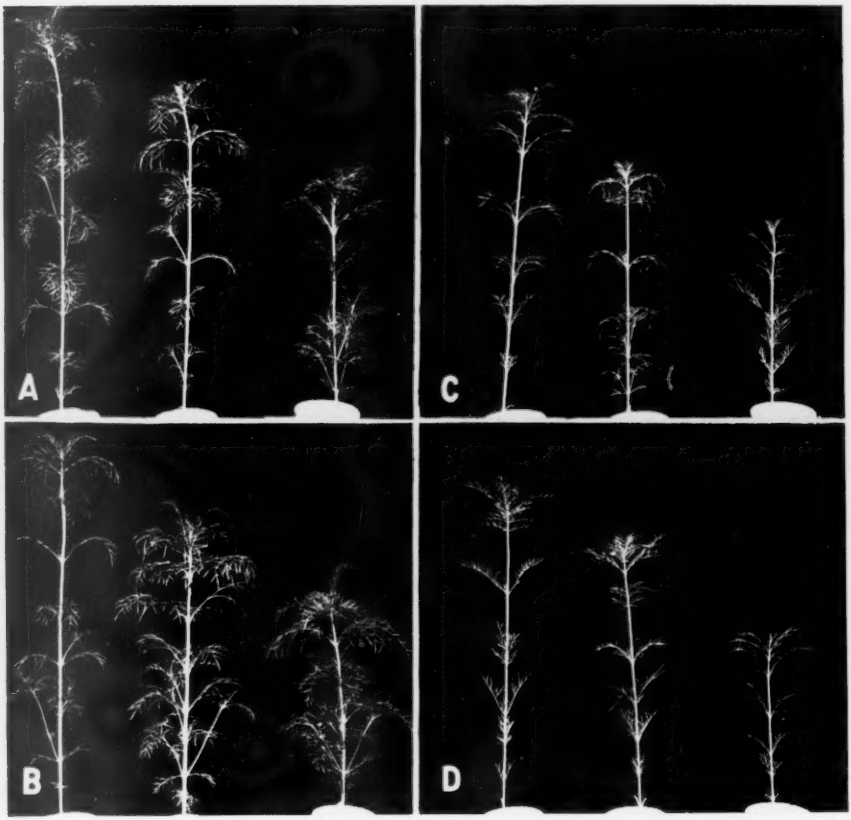

FIG. 2.—Early Single cosmos (experiment IV) grown on long photoperiod, photographed at time of harvest: *A*, grown with complete nutrient solution; *B*, similar to *A* but with vitamin B_1 added; *C*, similar to *A* but with one-fifth the nitrogen in the nutrient solution; *D*, similar to *C* but with B_1 added. Plants selected to show range of response to any specific treatment.

of light out of each 24-hour period. When the seedlings were large enough to handle, on March 30, they were selected for uniformity and transplanted—two each to $1\frac{1}{2}$-liter glazed crocks filled with quartz sand. Four hundred crocks were placed on each of two benches in the same greenhouse, 800 crocks in all. The plants on one bench were maintained on long photoperiod of 18 hours of daylight supplemented with light from 100-watt Mazda lamps; the other on short photoperiod, $8\frac{1}{2}$ hours of daylight out of each 24-hour period. The eight series of treatments given on opposite page were established, then divided into lots of ten plants each and completely randomized.

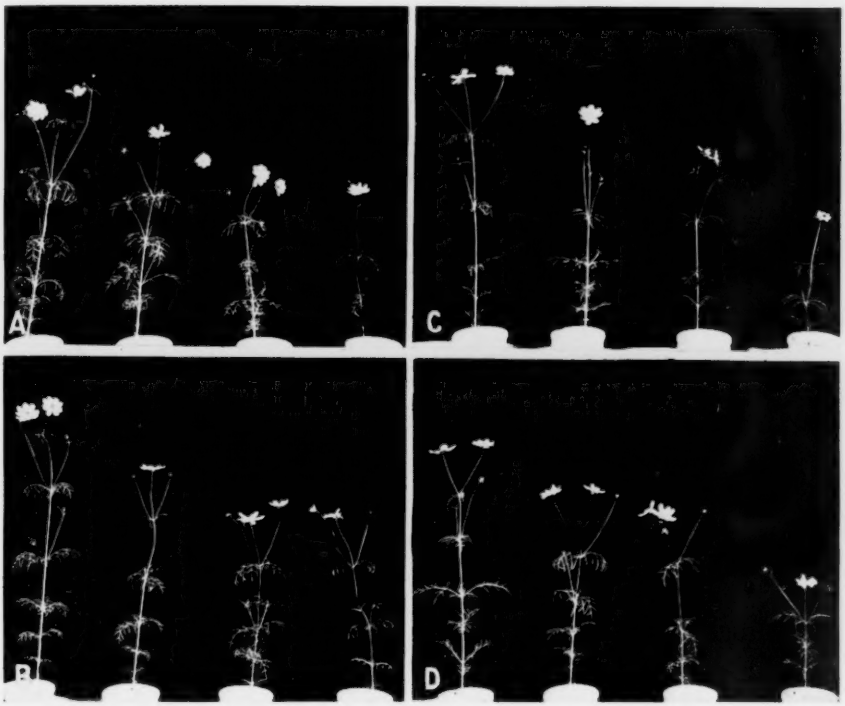

FIG. 3.—Early Single cosmos (experiment IV) grown on short photoperiod: *A*, with complete nutrient solution; *B*, similar to *A* but with vitamin B_1 added; *C*, similar to *A* but with one-fifth as much nitrogen in the nutrient solution; *D*, similar to *C* but with B_1 added. Plants selected on same basis of range of variation as in fig 2.

1. Long photoperiod, complete nutrient, supplied with vitamin B_1 each week.
2. Similar to 1, but with no B_1 added.
3. Similar to 1, but supplied with nutrient containing one-fifth as much nitrogen.
4. Similar to 3, but with no B_1 added.
5. Short photoperiod, complete nutrient, and supplied with B_1 once each week.
6. Similar to 5, but with no B_1 added.
7. Similar to 5, but supplied with nutrient containing one-fifth as much nitrogen.
8. Similar to 7, but with no B_1 added.

The plants were grown until those on short photoperiod had reached full bloom; they were harvested on May 13. The wet and dry weights of the tops and roots were taken. The plants were harvested using one row of ten plants as a unit; hence for each treatment ten weight determinations were made.

The cosmos plants, although selected for uniformity, showed wide range in growth response under any given treatment (figs. 2 and 3; tables 1, 2).

For experiment V, Burpee Golden cosmos was used. The experiment differed mainly from IV in that four series instead of eight were used, the series on the low plane of nitrogen supply being omitted. Also the short photoperiod was of $10\frac{1}{2}$ hours' duration instead of $8\frac{1}{2}$ hours, and the B_1 solution was supplied three times each week instead of once. The seeds were sown May 22, and the plants were harvested July 17 and July 18, at which time all the plants on short photoperiod were in full bloom, as were a few on long photoperiod (table 3).

Dahlia

Experiment VI.—On April 1, a large quantity of seeds of Coltness Gem dahlia were sown in soil. These germinated promptly, and on April 18, 200 plants were selected for uniformity and transplanted to waxed clay pots containing river sand. On May 17 all the plants were transferred to glazed crocks containing white quartz sand, care being used to disturb the roots as little as possible. All were supplied with nutrient solution; as in the other experiments, half of them received B_1 once each week. The plants grew vigorously and were harvested June 29 (table 4).

Mustard

Experiment VII.—On April 30, seedling plants of Giant Southern Curled mustard, germinated in white sand, were selected for uniformity and transplanted singly to waxed clay pots containing white quartz sand. The plants were randomized over a greenhouse bench and watered with complete nutrient solution; one half of them received B_1 once a week. The plants showed uniform growth throughout the experiment, and at no time did any striking differences between the two lots appear. They were harvested May 28 (fig. 4; table 4).

Radish

Three experiments were conducted: two in sand, and one in soil. In experiment VIII, seeds of French Breakfast were sown April 13 directly in wooden flats filled with river sand. After germination, the plants were thinned to approximately forty per flat. Eighteen flats were used, and the treatments were randomized over the bench. All the plants received the full nutrient, and one-half received in addition the B_1 once a week. The plants were harvested May 3 (fig. 1*C*, *D;* table 4).

Experiments IX and X were carried on concurrently. Flats were used and randomized as in experiment VIII. For experiment IX the variety Cardinal Globe was used. One-half the seeds were soaked for 2 hours in a 0.01 mg. per liter aqueous solution of vitamin B_1 and the other half in tap water. The two lots (ten flats of each) were sown separately May 7 in river sand, and the plants thinned as in

TABLE 1

DRY WEIGHT IN GRAMS OF EARLY SINGLE COSMOS PLANTS GROWING UNDER SHORT PHOTOPERIOD, WITH AND WITHOUT VITAMIN B_1 AND WITH COMPLETE NUTRIENT SOLUTION, AT HIGH AND LOW PLANES OF NITROGEN SUPPLY. EACH OF THE FOUR LOTS (WITH AND WITHOUT VITAMIN B_1) WAS RANDOMIZED ON THE BASIS OF 10 SAMPLES, EACH CONSISTING OF 10 PLANTS

	High nitrogen				Low nitrogen			
	With B_1		Without B_1		With B_1		Without B_1	
	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
	10.7	2.0	11.0	1.3	7.3	1.1	7.6	1.1
	11.1	1.6	11.7	1.6	7.8	1.2	7.7	1.5
	8.0	1.2	10.3	2.3	6.3	0.8	8.9	1.5
	8.7	1.1	9.2	1.1	7.4	1.3	7.0	1.0
	10.6	1.1	9.0	1.1	8.3	1.1	7.5	1.0
	9.2	1.1	9.6	1.3	7.6	1.0	7.6	1.4
	8.3	1.3	10.4	1.5	7.2	1.0	7.4	1.3
	11.9	1.4	10.1	1.0	7.0	1.1	8.8	1.5
	11.4	1.7	11.0	1.3	9.3	1.3	7.7	1.4
	11.1	1.9	11.2	1.4	7.3	1.1	7.7	1.4
Average:								
Per 10 plants	10.1	1.44	10.35	1.39	7.55	1.10	7.79	1.31
Per plant	1.01	0.13	1.03	0.139	0.75	0.110	0.779	0.131

TABLE 2

DRY WEIGHT IN GRAMS OF EARLY SINGLE COSMOS PLANTS GROWING UNDER LONG PHOTOPERIOD, WITH AND WITHOUT VITAMIN B_1 AND WITH COMPLETE NUTRIENT SOLUTION, AT HIGH AND LOW PLANES OF NITROGEN SUPPLY. EACH OF THE FOUR LOTS (WITH AND WITHOUT VITAMIN B_1) WAS RANDOMIZED ON THE BASIS OF 10 SAMPLES, EACH CONSISTING OF 10 PLANTS

	High nitrogen				Low nitrogen			
	With B_1		Without B_1		With B_1		Without B_1	
	Tops	Roots	Tops	Roots	Tops	Roots	Tops	Roots
	41.0	10.6	40.0	13.9	17.2	5.6	17.9	7.1
	35.0	16.7	32.5	11.5	18.2	6.4	16.9	5.9
	42.5	11.9	36.9	9.8	19.6	6.2	19.4	6.1
	39.7	10.0	37.8	10.8	17.6	6.6	15.8	5.9
	40.5	11.0	38.3	10.6	16.9	5.8	16.8	6.6
	40.3	10.6	43.1	11.1	16.9	5.7	14.4	3.7
	43.5	11.4	38.5	9.0	16.5	5.8	17.2	5.9
	43.1	10.5	39.8	10.5	19.1	5.9	18.0	4.9
	40.6	9.4	42.5	11.6	19.5	6.1	18.0	5.6
	41.3	10.2	36.3	8.3	17.9	5.1	16.4	4.6
Average:								
Per 10 plants	40.75	11.23	38.57	10.71	17.94	5.92	17.08	5.63
Per plant	4.07	1.12	3.85	1.07	1.79	0.59	1.70	0.56

experiment VIII. All were watered with full strength nutrient solution and with tap water. The lot of plants from the seeds soaked in B_1 solution received in addition the B_1 once a week. The plants were harvested June 3.

For experiment X the variety Crimson Giant was used. The seeds were soaked in the same way as in experiment IX, but they were sown in flats of garden loam instead of river sand, on May 7. Two lots of nine flats each were randomized as usual, and watered with tap water. To the plants from the seeds steeped in B_1

TABLE 3

DRY WEIGHT IN GRAMS OF BURPEE GOLDEN COSMOS PLANTS GROWING UNDER LONG PHOTOPERIOD, WITH AND WITHOUT VITAMIN B_1 AND SHORT PHOTOPERIOD WITH COMPLETE NUTRIENT. EACH OF THE FOUR LOTS (WITH AND WITHOUT VITAMIN B_1) WAS RANDOMIZED ON THE BASIS OF 11 SAMPLES, EACH CONSISTING OF 10 PLANTS

	LONG PHOTOPERIOD				SHORT PHOTOPERIOD			
	WITH B_1		WITHOUT B_1		WITH B_1		WITHOUT B_1	
	TOPS	ROOTS	TOPS	ROOTS	TOPS	ROOTS	TOPS	ROOTS
	47.0	9.9	48.0	8.5	26.5	3.2	25.5	3.0
	49.5	9.6	50.0	11.1	39.5	4.8	41.5	5.8
	51.5	9.3	55.5	10.1	35.5	5.0	39.0	6.4
	44.0	9.8	48.5	9.9	36.0	4.9	28.0	3.9
	46.0	11.2	66.0	12.0	43.5	5.3	39.0	5.7
	52.5	9.7	47.0	9.5	41.5	6.2	35.0	5.0
	56.5	11.7	53.0	10.6	36.5	6.1	40.0	6.0
	53.0	10.8	57.5	12.4	36.5	5.3	40.5	5.6
	53.5	9.6	48.5	12.3	43.0	5.9	40.0	5.7
	47.5	9.2	51.5	10.5	40.5	5.7	39.0	4.9
	46.0	8.0	73.5	13.2	34.0	5.0	42.0	6.1
Total	547.0	108.8	599.0	120.1	413.0	57.4	409.5	58.1
Average								
Per 10 plants	49.72	9.9	54.45	10.91	37.4	5.21	37.22	5.28
Per plant	4.97	0.99	5.45	1.09	3.74	0.521	3.72	0.528

solution the usual B_1 solution was added once each week; the others received tap water only. The plants were harvested June 3.

In all the experiments on radish no significant differences were observed (table 4).

ZINNIA

EXPERIMENT XI.—On April 10, 2000 seeds of Star Dust variety of zinnia were planted in quartz sand. After germination, plants were re-selected for uniformity and on April 22 about 500 were transplanted singly into 4-inch waxed clay pots. Two hundred and twenty-four were again selected for uniformity and randomized on a greenhouse bench in rows of eight. All the plants were watered with nutrient

TABLE 4

EXPERIMENTS ON SEVERAL VARIETIES OF PLANTS, SPECIFIC TREATMENTS AS INDICATED; WEIGHT IN GRAMS

Variety of plant	No. of experiment	Specific treatment	No. of plants used	No. of random samples	Average dry weight per plant (gm.)	
					Tops	Roots
Cabbage (Early Jersey Wakefield)	I	Grown in sand with complete nutrient:				
		B_1 added	104	13	5.36	1.23
		Control*	104	13	5.29	1.30
Cocklebur	II	In sand with complete nutrient on long photoperiod:				
		B_1 added	95	19	4.24	1.11
		Control	95	19	4.18	1.09
Cocklebur	III	In sand with complete nutrient on long photoperiod:				
		B_1 added	180	18	3.68	0.90
		Control	180	18	3.64	0.92
		Transferred to short photoperiod for 2 weeks just prior to harvesting				
		B_1 added	200	20	3.85	0.72
		Control	200	20	3.81	0.72
Cosmos (Early Single)	IV	(See tables 1 and 2)	.....	.....	.....	.....
Cosmos (Burpee Golden)	V	(See table 3)	.....	.....	.....	.....
Dahlia (Coltness Gem)	VI	In sand with complete nutrient solution:				
		B_1 added	91	13	6.36	3.41
		Control	91	13	6.46	3.10
Mustard (Giant Southern Curled)	VII	In sand with complete nutrient solution:				
		B_1 added	88	11	2.67	0.53
		Control	88	11	2.68	0.53
Radish (French Breakfast)	VIII	In sand with complete nutrient solution:				
		B_1 added	432	9	0.205	0.153
		Control	432	9	0.210	0.156
Radish (Cardinal Globe)	IX	In sand with complete nutrient solution:				
		B_1 added	320	8	0.289	0.107
		Control	320	8	0.313	0.105
Radish (Crimson Giant)	X	In soil:				
		B_1 added	312	8	0.478	0.232
		Control	312	8	0.508	0.233
Zinnia (Star Dust)	XI	In sand with complete nutrient solution:				
		B_1 added	112	14	1.96	0.38
		Control	112	14	1.95	0.38

* No. B_1 added to culture solution or soil.

solution, and one-half of them were given in addition the B_1 solution once each week. All the plants grew vigorously but showed considerable range of variation, irrespective of treatment given. They were harvested on June 3 (table 4).

FIG. 4.—Giant Southern Curled mustard (experiment VII): *A*, control; *B*, with vitamin B_1 added. Photographed at time of harvest.

Discussion

At the present time it is not possible to account for the discrepancies between the results of the experiments reported here and those of BONNER and GREENE (2, 3) and of BONNER (1). The individual plants in the present experiments were many times larger and very much heavier than those reported by them. For example, the average weight per plant of the tops in grams of cosmos plants grown by BONNER and GREENE under long photoperiod for a period of 8 weeks was 0.0675

gm. for the controls and 0.110 gm. for the plants to which B_1 had been added. In the present experiment with Early Single cosmos, the average weight per plant under long photoperiod for 6 weeks was 3.85 gm. for the controls and 4.07 gm. for those to which B_1 had been added in the culture medium. In the case of Burpee Golden cosmos the differences were still greater. Similarly, in experiments on cocklebur grown on long photoperiod for 4 weeks and which were 7 weeks old when harvested (1), the weight of the tops of control plants averaged 0.810 gm. and those to which B_1 had been added, 1.00 gm. In the experiments reported here, the average weight per plant of cocklebur grown under similar conditions of photoperiod was four times as great. Little difference was shown between the controls and those to which B_1 had been added.

As already stated, the pH of the nutrient solution was 4.8; alkaline conditions never prevailed throughout the entire length of any experiment. The solutions of vitamin B_1 were always prepared immediately before use.

In the cases of Early Single cosmos, cocklebur, zinnia, and radish the range of variation among the plants of the several varieties was large. It is certainly true, as the detailed figures and the illustrations clearly show, that variation within experimental lots was often as great under any given set of conditions imposed as between different experimental conditions. Because of this fact, large populations, thoroughly randomized, were always used. In the case of cabbage and mustard, the plants were strikingly uniform in appearance in any given lot and showed little difference between controls and those to which B_1 had been added.

To say that the addition of vitamin B_1 is of particular value in increasing growth or dry weight accumulation when added to plants of any of the varieties or species tried—when they are subjected to growth limitation through photoperiod or nutrient supply—is not in keeping with the results of these experiments. There is marked difference between the lots of plants grown on long photoperiod as contrasted with those grown on short photoperiod, on a high plane of nitrogen supply as compared with those on a low one; but the addition of vitamin B_1 in no way changed this relationship. Statistically the differences which could be ascribed to the addition of B_1 as compared with a control under similar environment were never significant.

Summary

1. Vitamin B_1 was added to a number of different plants in a concentration of 0.01 mg. per liter. In most of the experiments the plants received vitamin B_1 in the nutrient solution once a week; however, one of the cocklebur experiments received vitamin B_1 twice a week, and in one of the cosmos experiments vitamin B_1 was added three times a week.

2. No visible differences were detected between the control plants and those re-

ceiving B_1. No significant difference was obtained in wet weight or in the accumulation of dry matter.

3. Under neither a long nor a short photoperiod, nor at high nor low planes of nitrogen nutrition, were there detectable differences which could be ascribed to additions of vitamin B_1 to the cultures.

4. Vitamin B_1 had no effect upon hastening flowering, upon size or number of flowers produced, or upon color or quality of the flowers of cosmos.

DEPARTMENT OF BOTANY
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RESPONSES OF MARCHANTIA POLYMORPHA TO NUTRIENT SUPPLY AND PHOTOPERIOD[1, 2]

CONTRIBUTIONS FROM THE HULL BOTANICAL LABORATORY 515

PAUL D. VOTH AND KARL C. HAMNER

(WITH FOURTEEN FIGURES)

Introduction

As experimental material in studies on plant nutrition, *Marchantia polymorpha* L. has been used for many years. Various substrates have been used, including soil (**5, 29, 31, 33**), agar with various nutrients (**6, 8, 10, 23, 34**), blotting or filter paper (**5, 6, 29**), sand (**29**), cinders (**38**), peat (**29, 31**), and nutrient solutions (**1, 5, 9, 10, 39**). These investigations have utilized gemmae in the main and include studies of dorsiventrality (**1, 5, 8, 29, 31, 39**), effect of the concentration and composition of nutrient solution (**1, 2, 10, 11, 23, 33**), effect of temperature variations (**1, 5, 9, 10, 11**), intensity and quality of light (**5, 6, 8, 9, 10, 11, 23, 34, 39**), and photoperiod (**5, 38**). Work on seed plants (**17, 28**) has indicated a possible interrelationship between mineral nutrition and photoperiod. The present studies deal principally with similar relationships in *Marchantia*, with particular reference to the development of the gametophyte and gametangiophores.

Marchantia grows readily on moist soil, but our attempts to grow it in sand culture have been more or less unsuccessful because of the tendency of the surface of the sand to become dry, and consequently the plants do not obtain sufficient moisture. When a system of continuous irrigation in sand was used, salts tended to accumulate on the surface of the sand, resulting in injury to the plants. Most of these difficulties were avoided by the use of methods subsequently described in this paper. Plants were grown on a wide variety of nutrient solutions, under various conditions of photoperiod. Records were made in each case of changes in the gross appearance of the plants, increase in total area, formation of gemmae cups and gametangiophores, anatomical variations, and the accumulation of dry weight.

General methods

The strains of *Marchantia polymorpha* used in these experiments produce (as usually grown) antheridiophores in late February and early March and archegoniophores a few weeks later. Gemmae cups are produced abundantly in autumn

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and winter. The experiments described here were conducted in the greenhouses at the University of Chicago during the late spring, summer, and fall of 1939 and the spring of 1940.

Sand culture having proved unsatisfactory, floats of balsa wood were devised to support the thallus and to keep it in contact with the surface of the culture solu-

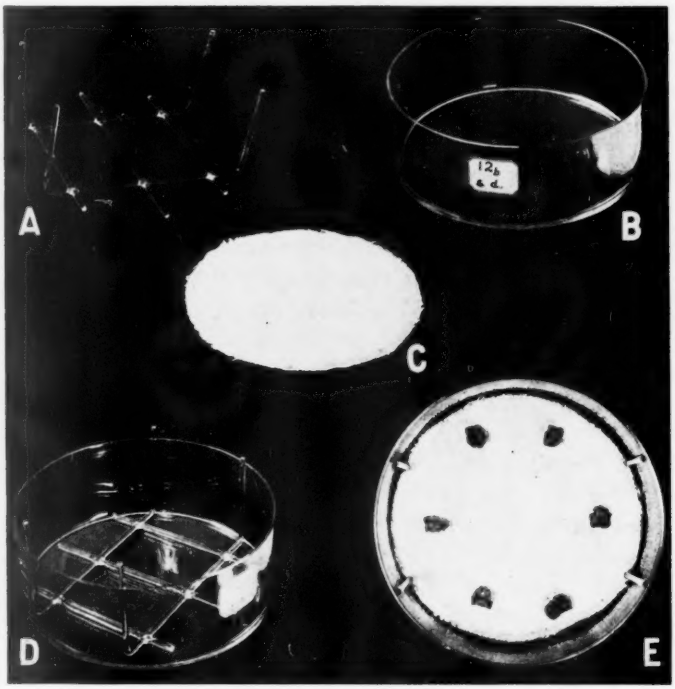

FIG. 1.—Apparatus used in growing *Marchantia: A*, glass rack or framework welded from pieces of glass rod, 5 mm. in diameter; *B*, half a glass moist chamber, approximately 9×3 inches; *C*, glass cloth; *D*, glass rack in moist chamber; *E*, complete assembly with six cuttings.

tion. This method was abandoned because the floats tended to become water logged, even though impregnated with paraffin.

After several preliminary trials, it was found that *Marchantia* would grow well if placed upon a coarse grade of glass cloth in contact with nutrient solution. The cloth served as a wick and was moist throughout its area if kept in contact with the solution at one or two points. The setup used is illustrated in figure 1. Half of a moist chamber was placed on a greenhouse bench with the open face upward. Glass rods were welded together to form a circular framework which would just fit into the chamber, and over this framework a circular piece of glass cloth was so placed that it was approximately $\frac{1}{4}$ inch above the bottom of the chamber.

Sufficient culture solution was added to come into contact with the lower surface of the cloth. Extensions of the two longer rods of the framework were bent upward to serve as handles in facilitating removal from the moist chamber. Before use in establishing a culture in any experiment, the entire setup was thoroughly cleansed by treatment for 24 hours in cleaning solution and then thoroughly washed in tap water, followed by distilled water. All nutrient solutions were changed every day or every other day, depending upon the temperature and the amount of evaporation. Every second day the framework and glass cloth were removed and rinsed thoroughly in distilled water, and fresh nutrient solution was again placed immediately in the moist chamber.

Using this method, an attempt was made to grow *Marchantia* on a solution which had an osmotic concentration of approximately 0.79 atmospheres. At full strength this solution consisted of 12 cc. of 0.5*M* $Ca(NO_3)_2$, 9 cc. of 0.5*M* KH_2PO_4, 9 cc. of 0.5*M* $MgSO_4$, and 1 p.p.m. of some of the microelements—except $FeSO_4$, which was supplied at 0.1 p.p.m. (**16**). The plants grew fairly well in this solution when compared with similar plants grown on soil. There was, however, some evidence of salt excess in the nutrient cultures; consequently, several experiments were conducted in which the growth of *Marchantia* on the full strength solution was compared with its growth on dilutions of one-fifth and one-tenth the original concentration. Control plants were grown on soil, tap water, and distilled water. Responses to the one-tenth dilution were similar to the one-fifth dilution. Because of the growth responses, production of gametangiophores and gemmae cups, and area of thallus developed, the concentration of solution used as a basis in making up the nutrient combinations in all subsequent experiments was comparable to that of the one-fifth dilution of the solution originally employed. It is not known whether the greater growth was owing to a lower total osmotic concentration or to a lower concentration of certain of the individual inorganic salts. In all subsequent work the solutions had an osmotic concentration of approximately 0.285 atmospheres.

A range of nutrient solutions was used according to the method reported by C. L. Hamner (**16**). Six stock solutions were prepared with molal concentrations as given in table 1. Each of three of these contained only K, Ca, or Mg as the cation, but all contained the three anions NO_3, PO_4, or SO_4. Each of the other three contained only NO_3, PO_4, or SO_4 as the anion, but had the cations K, Ca, and Mg. This was accomplished by the use of nine salts, as indicated in table 1. By the use of these solutions, and by effecting various combinations of them on the basis of sixths, it was possible to arrange a triangle of twenty-eight different solutions with special reference to the cations present and an additional twenty-eight with particular reference to the anions. The combinations composing each triangle were placed in order on the basis of their relative composition and

numbered (fig. 2). The twenty cultures on short photoperiod were supplied with solutions such as occurred only in the central portion of the triangle, ten representing the cations and ten the anions. The pH of all stock solutions (table 1) and those supplied the plants (fig. 2) was between 5.0 and 6.0. All solutions became neutral or slightly alkaline after being in the moist chambers in contact with the growing plants for a day, except the solutions lacking in nitrogen, which changed very little.

The conditions for long photoperiod were obtained by means of 200-watt Mazda lamps with reflectors suspended about 3 feet above the greenhouse bench. These lamps burned from sundown until 2:00 A.M. each night, supplying a maximum illumination of approximately 100 foot-candles at the surface of the plant.

TABLE 1

CONCENTRATIONS OF SALTS IN THE 6 STOCK NUTRIENT SOLUTIONS USED IN MAKING UP 56 OTHER COMBINATIONS. TO EACH STOCK SOLUTION WAS ADDED 0.2 P.P.M. OF $MnSO_4$, $ZnCl_2$, AND $Na_2B_4O_7$, AND ALSO 0.02 P.P.M. OF $FeSO_4$

SALT	MOLAL CONCENTRATIONS OF INDIVIDUAL STOCK SOLUTIONS					
	K	Ca	Mg	NO_3	PO_4	SO_4
KNO_3	0.0024			0.0009		
KH_2PO_4	0.0009				0.0009	
K_2SO_4	0.0009					0.00045
$Ca(NO_3)_2$		0.0012		0.0012		
$Ca(H_2PO_4)_2$		0.00045			0.0012	
$CaSO_4$		0.0009				0.0012
$Mg(NO_3)_2$			0.0012	0.0009		
$MgHPO_4$			0.0009		0.0009	
$MgSO_4$			0.0009			0.0009

The short photoperiod was obtained by building a framework over the bench and covering it completely with black cloth at 5:00 P.M. every evening. The black cloth was removed at 8:00 A.M. each morning. Thus the plants exposed to long photoperiod received approximately 18–20 hours of light out of each 24-hour period, and plants exposed to short photoperiod received 9 hours of light.

All the plants were propagated vegetatively from a single male plant and a female plant. They were grown in the greenhouse on a one-fifth dilution of a three salt solution (**16**) and furnished the stock from which the plants used for experimentation were taken. At the beginning of any particular experiment, a small portion from the tip of a branch of the stock was taken. Six of these cuttings were selected for uniformity and arranged equidistantly on the glass cloth (fig. 1*E*). The growing point of each cutting was directed toward the center of the dish. At the time of making the cuttings, the total area of each particular group of six was determined by means of an area photometer (**24**). Several representative

groups of six cuttings designated as initial controls were also set aside for fresh and dry weight determinations, and other representative cuttings were fixed for subsequent sectioning in a formalin-acetic-alcohol solution consisting of 67 cc. forma-

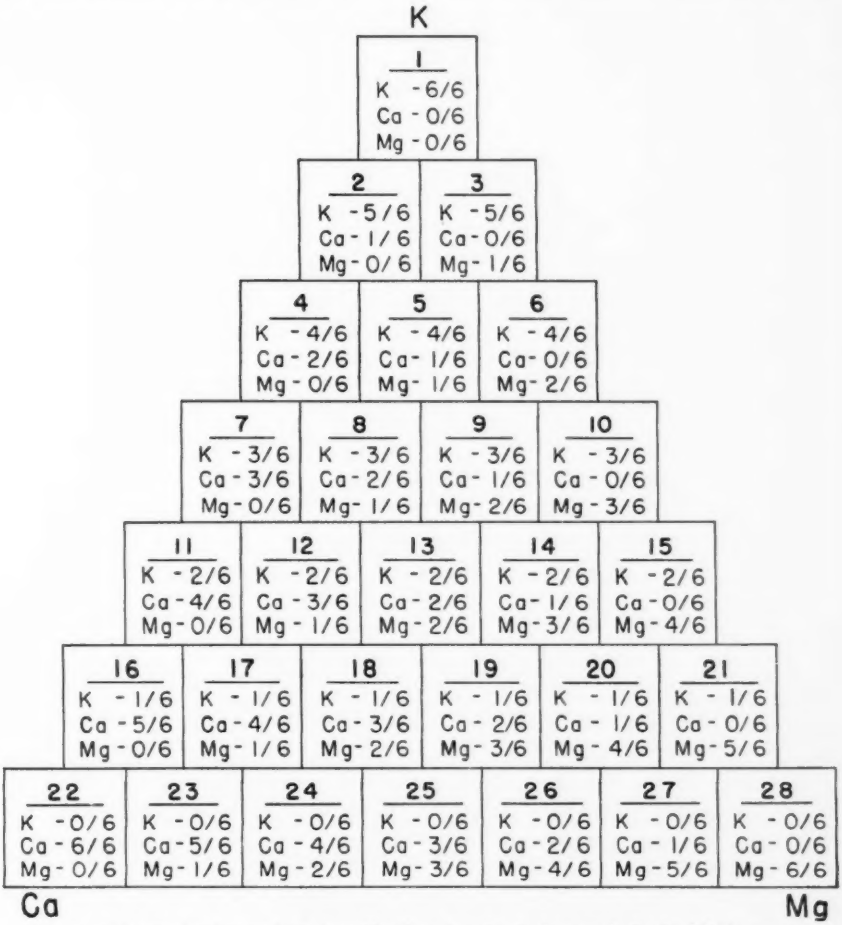

FIG. 2.—Triangle showing method of combination of stock solutions on the basis of sixths, to secure a range of 28 solutions of varying cations. Each apical position lacks two cations; each side of the triangle lacks one; and the center is supplied with all three in varying proportions. A triangle for varying the combination of anions could be effected in a similar manner.

lin, 23 cc. glacial acetic acid, 500 cc. of 95 per cent alcohol, and water sufficient to make 1 liter.

At the completion of an experiment, the plants were carefully loosened from the glass cloth by means of a sharp spatula, each group was photographed, and the

total area was determined. Careful notes were made as to the number of gemmae cups, the number of antheridiophores or archegoniophores, and the general appearance of the plants. The fresh weight of the plants was then determined, and one plant selected for fixation in F.A.A. and subsequent sectioning. The fresh weight of the remaining five plants was determined. They were then dried at 102° C. On the basis of the percentage dry weight of five plants, and the fresh weight of the six plants, the total dry weight of six plants was calculated.

Investigation

Details of specific experiments

Experiment 8.—*On March 14, 1940, fifty-six cultures were placed under conditions of long photoperiod and twenty under short photoperiod.* The six cuttings in each culture totaled 15 sq. cm. in area. In any set of six plants there were approximately 16–18 growing points. Four more cultures served as initial controls. The fresh and dry weights of each lot varied very little. The average dry weight was 60 mg. The character of growth, development, and final results obtained under conditions of short photoperiod are described later in a special section. The growth and general appearance of the plants subjected to conditions of long photoperiod at progressive dates were as follows.

On March 22, 8 days after beginning the experiment, progressive death was noted in certain of the cultures in the cation triangle on long photoperiod. The growing points of the plants supplied with nutrient solutions lacking in calcium were dead, and necrosis in the wings of the thallus followed. The most rapid development of this symptom occurred in nutrient solution 28. The other solutions lacking calcium ranged in this order of toxicity: 21, 15, 10, 6, 3, and 1. While all these solutions are lacking in calcium, they contain various proportions of magnesium and potassium. Plants on the solutions relatively high in magnesium and low in potassium seemed to develop the calcium deficiency symptoms more rapidly than did those on solutions high in potassium and low in magnesium. The first evidence of calcium deficiency was darkening of the growing point, which later became translucent, then very black, and finally died completely. Fourteen days after the start of the experiment, or March 28, the calcium deficiency symptoms were much more apparent. On April 2, growing points of all the plants supplied with a nutrient solution lacking calcium were dead, and portions of the thallus were disintegrated. On April 16 there was some regeneration in the plants growing on solutions lacking in calcium. This was particularly true with the plants on solutions 1, 3, 6, and 10. Slight regeneration was apparent in the plants on solution 15, and those plants on solutions 21 and 28 were apparently dead.

There were no particular symptoms of magnesium deficiency on April 2, except that the plants on solutions lacking magnesium grew less than some of the others.

The greatest growth in the cation triangle was made by plants receiving solutions 8, 11, 12, 13, 17, and 18. The plants supplied with nutrient solutions lacking potassium did not grow so well, and the older portions of the thallus of such plants had died. This portion of the thallus was often straw colored rather than bright green. On April 16, approximately 50 per cent of the base of each plant supplied with solutions lacking in potassium was dead. There was very little evidence of magnesium deficiency in any of the plants at this time, except perhaps that the plants on solutions lacking magnesium did not grow quite so rapidly. No evidence of potassium or calcium excess symptoms was recorded. There may have been some effect of excess in magnesium, as evidenced by the fact that plants on solution 28 were apparently dead early in the course of the experiment. Plants on solutions 21 and 15 also appeared dead by the time of harvest. The fact that solutions 21 and 15 were not so favorable for growth as were solutions 1 and 3 may have been owing to the presence of a higher concentration of magnesium in solutions 21 and 28 than in solutions 1 and 3, or possibly to the lower concentration of potassium in the former as compared with the latter. All these solutions, of course, contained no calcium.

By March 28, certain types of growth responses were evident in the plants of the anion triangle exposed to long photoperiod. Those plants growing on solutions lacking phosphorus were somewhat greener than the others, and certain of them developed a green-black color in the midrib region. By April 2, all the plants on nutrients lacking phosphorus had black midribs. At this time the greatest growth in the anion triangle was on solutions 31, 33, 34, 36, 37, and 38. Up to this time absence of phosphorus had not resulted in appreciable decrease in the rate of growth, if at all. On April 16, the plants on solutions lacking phosphorus all exhibited extensive growth and were of darker green color than plants on any of the other solutions. Particularly the regions near the tips of the thallus were dark green, the midrib was black or very deep red, and the bases of the plants were tan. On this date, those plants growing on solutions lacking nitrate were stunted, producing much less growth than those on other solutions. They had a red coloration which at first was ascribed to anthocyanin in the scales on the ventral surface of the thallus. This red color and the color of the plants lacking in phosphorus will be discussed in the anatomical section. The margins of the thalli possessed very little chlorophyll and were almost white. Those plants growing on solutions lacking in sulphate produced slightly less growth than those on some of the other solutions. There was some evidence of phosphate excess in plants growing on solutions 50, 44, 51, 39, 45, and 52. The margins of these plants were necrotic and subsequently tended to dry up, as if burned. This symptom, however, was not very marked at any time.

All plants were harvested on April 16 (figs. 3, 4, 7, 8). The records of the num-

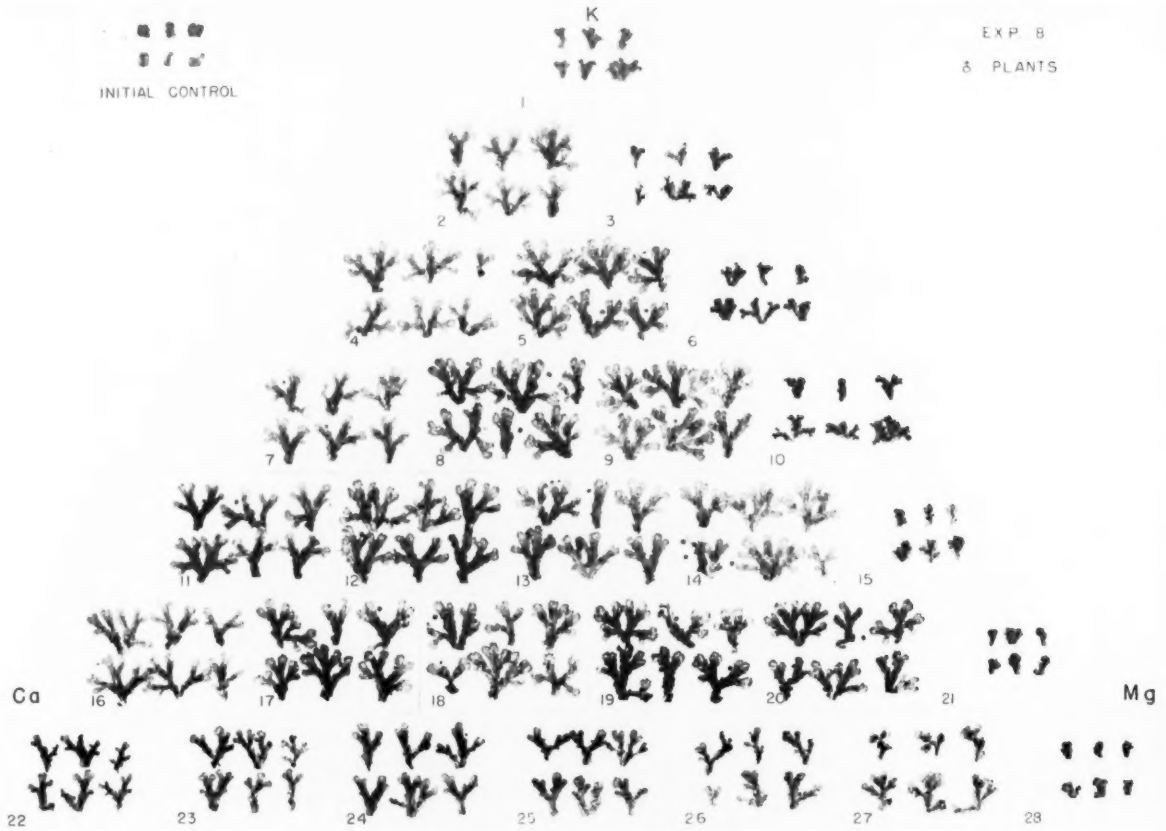

FIG. 3.—Cation triangle of experiment 8, April, 1940; antheridial plants on long photoperiod

FIG. 3.—Cation triangle of experiment 8, April, 1940; antheridial plants on long photoperiod

FIG. 4.—Anion triangle of experiment 8, April, 1940; antheridial plants on long photoperiod

ber of gemmae cups and of antheridiophores are given in tables 2 and 3. Certain of the plants possessed only a few gemmae cups, and these occupied a position in

TABLE 2

GROWTH OF MARCHANTIA POLYMORPHA ON NUTRIENT SOLUTIONS CONTAINING VARIOUS PROPORTIONS OF CATIONS OR ANIONS. ALL PLANTS GROWN ON LONG PHOTOPERIOD

Position in triangle	Weight of 6 plants (gm.)				Rank of various solutions in each triangle on basis of dry weight of 6 plants		Area of 6 plants (sq. cm.)		No. of gemmae cups on 6 plants		No. of gametangiophores on 6 plants	
	Exp. 8		Exp. 9									
	Fresh	Dry	Fresh	Dry	Exp. 8	Exp. 9	Exp. 8	Exp. 9	Exp. 8	Exp. 9	Exp. 8 Antheridiophores	Exp. 9 Archegoniophores
	Cations K, Ca, and Mg varied proportionately on basis of sixths (cf. table 1) Anions present in constant relative proportion											
1	2.88	0.257	2.72	0.095	25	28	47	34	3	0	0	0
2	8.69	0.587	6.32	0.356	14	21	158	103	81	0	4	3
3	3.32	0.283	3.51	0.302	24	22	50	60	9	0	1	4
4	7.06	0.581	7.23	0.523	15	14	145	129	50	6	15	1
5	13.36	0.780	19.94	0.917	4	10	186	324	126	66	16	1
6	3.40	0.309	4.38	0.272	23	25	55	70	0	0	4	1
7	7.76	0.601	7.23	0.493	13	17	137	122	27	1	9	4
8	12.93	0.848	21.38	1.155	1	4	201	375	126	9	7	0
9	12.42	0.737	21.27	1.046	7	8	230	381	104	21	8	2
10	3.49	0.317	6.95	0.487	22	18	65	105	2	7	3	4
11	9.07	0.746	6.82	0.607	6	13	161	134	27	0	6	10
12	11.55	0.813	18.62	1.123	3	5	219	315	86	3	1	0
13	10.48	0.678	16.53	0.980	10	9	184	286	97	6	8	5
14	10.21	0.667	23.54	1.314	11	2	200	442	83	41	4	2
15	1.71	0.153	3.38	0.273	26	24	40	56	2	0	0	2
16	8.15	0.703	7.86	0.619	9	12	167	136	2	0	1	8
17	10.45	0.825	16.18	1.060	2	7	197	283	47	0	11	6
18	10.46	0.718	18.75	1.123	8	6	178	374	57	0	12	5
19	11.16	0.763	27.33	1.741	5	1	192	496	85	14	5	4
20	8.76	0.654	21.33	1.297	12	3	170	370	58	13	6	7
21	1.27	0.132	2.51	0.200	27	26	25	37	1	0	1	1
22	3.68	0.337	2.54	0.283	20	23	73	36	5	0	3	6
23	4.70	0.403	5.09	0.473	18	19	95	87	3	0	1	4
24	5.76	0.477	7.58	0.697	16	11	112	141	9	0	6	7
25	4.02	0.404	5.83	0.494	17	16	97	105	0	4	8	0
26	3.49	0.328	7.27	0.518	21	15	80	134	9	2	11	4
27	4.18	0.339	5.00	0.423	19	20	87	94	28	0	10	0
28	1.00	0.102	1.94	0.164	28	27	25	25	0	0	0	0
Totals	.....	.....	.....	.....	.....	.....	3576	5254	1127	193	161	91

the older portion of the thallus and may have been differentiated at the time the cutting was made. Other plants possessed a large number, and most of these were present in the younger areas. A discussion of the results is deferred for comparison with the results of experiment 9.

EXPERIMENT 9.—On April 26, an experiment using cuttings from archegonial plants as described for experiment 8 was begun.

TABLE 2—*Continued*

POSITION IN TRIANGLE	WEIGHT OF 6 PLANTS (GM.)				RANK OF VARIOUS SOLUTIONS IN EACH TRIANGLE ON BASIS OF DRY WEIGHT OF 6 PLANTS		AREA OF 6 PLANTS (SQ. CM.)		NO. OF GEMMAE CUPS ON 6 PLANTS		NO. OF GAMETANGIOPHORES ON 6 PLANTS	
	EXP. 8		EXP. 9									
	FRESH	DRY	FRESH	DRY	EXP. 8	EXP. 9	EXP. 8	EXP. 9	EXP. 8	EXP. 9	EXP. 8 ANTHERIDIOPHORES	EXP. 9 ARCHEGONIOPHORES
	ANIONS NO_3, PO_4, AND SO_4 VARIED PROPORTIONATELY ON BASIS OF SIXTHS (CF. TABLE 1) CATIONS PRESENT IN CONSTANT RELATIVE PROPORTION											
29	10.48	0.755	12.26	0.720	8	14	196	202	96	89	21	4
30	12.52	0.795	33.66	1.861	6	1	194	532	52	30	13	1
31	9.91	0.745	12.79	0.794	11	13	183	234	96	97	16	4
32	12.59	0.806	21.62	1.142	5	5	210	370	62	22	8	3
33	14.38	1.012	29.06	1.755	1	2	230	507	69	18	11	0
34	10.10	0.727	14.21	0.926	15	9	205	252	105	50	31	16
35	8.86	0.577	8.83	0.529	19	20	170	167	11	1	2	4
36	12.80	0.837	15.63	0.880	4	10	236	284	62	0	11	5
37	11.15	0.919	21.91	1.258	2	4	234	401	64	10	19	2
38	9.56	0.738	16.17	1.069	13	7	197	280	86	6	19	6
39	10.94	0.686	14.50	0.874	16	11	142	250	20	1	10	5
40	8.78	0.753	13.29	0.811	9	12	175	264	47	0	14	5
41	8.55	0.610	17.61	1.090	18	6	173	325	29	0	8	8
42	11.72	0.745	22.36	1.353	10	3	244	405	75	0	5	10
43	11.13	0.851	8.72	0.523	3	21	220	172	84	48	30	0
44	7.48	0.513	11.30	0.678	21	16	150	174	22	0	16	10
45	9.50	0.740	8.08	0.595	12	19	208	165	25	0	7	8
46	9.28	0.629	9.96	0.689	17	15	204	188	14	0	5	11
47	10.31	0.734	9.33	0.596	14	18	211	189	34	1	6	19
48	10.32	0.783	14.57	1.039	7	8	215	288	64	0	11	10
49	7.21	0.559	9.07	0.601	20	17	158	174	44	2	18	11
50	3.23	0.265	3.05	0.220	25	24	80	47	4	0	9	3
51	3.41	0.230	3.35	0.219	26	25	88	50	2	0	7	14
52	2.93	0.206	2.57	0.181	28	27	75	42	2	0	5	6
53	3.09	0.222	2.38	0.172	27	28	80	44	2	0	4	2
54	3.46	0.286	2.57	0.204	24	26	77	48	1	0	9	7
55	3.98	0.343	2.95	0.234	22	23	90	71	1	0	15	4
56	3.63	0.331	3.18	0.250	23	22	80	67	0	0	15	5
Totals							4725	6192	1173	375	345	183

On May 19, the plants under conditions of long photoperiod and supplied with solution 1 were watery in appearance, and some of them became infected with molds. Most of these plants were practically dead at this time. These symptoms may be attributable to the presence of a high concentration of potassium or to the combined absence of magnesium and calcium. On this same date, the plants

supplied with solution 22 were much smaller than most of the other plants and were dark green in color, streaked with black or deep red along the midribs. The older portion of the thallus was streaked with yellow-green. The plants supplied with solution 28 had grown very little up to this date, were black in color, and all were dead. Plants supplied with solution 21 had some of the symptoms apparent in those plants supplied with solution 28, although they had not progressed so far. The plants supplied with solution 15 showed some symptoms similar to those developed in 21 and 28. Apparently, if calcium is lacking, a high concentration of magnesium—together with a low concentration of potassium—is definitely toxic. Those plants supplied with solutions lacking in potassium (that is, supplied with solutions 22 to 28, inclusive) developed characteristic symptoms. The margins of the older portion of the thallus turned light tan to brown, finally becoming watery in appearance, and ultimately the margins became dry. Progressive stages of this symptom were characterized by concentric rings, each ring marked by a slightly darker brown color, particularly noticeable in the notches between the branches of the thallus. Those plants supplied with solutions lacking in calcium (solutions 1, 3, 6, 10, 15, 21, and 28) developed more or less characteristic symptoms, as described for experiment 8. In this particular group, plants on solution 10 grew the most. There was some evidence of regeneration of the thallus.

Those plants supplied with solutions lacking in magnesium (solutions 1, 2, 4, 7, 11, 16, and 22) did not develop any characteristic symptom. Plants supplied with solution 22 produced very little growth, and there was some burning in the margins of the plants supplied with solutions 2, 4, 7, 11, and 16. The thallus of plants supplied with solution 1 had extensive burning at the margins and grew very slightly. On this date all the plants in the central portion of the triangle were about equal in gross appearance.

On May 19, the plants supplied with solutions of the anion triangle were also carefully examined. Those supplied with solutions lacking in nitrates (solutions 50 to 56, inclusive) were all small and had produced only enough growth approximately to double the original area of the cuttings. All these plants were pale yellow-green in color, and the scales on the ventral surface were bright red. This color could be seen showing through the more or less transparent thallus. The tips of the thalli were light green, and branches were somewhat narrow at the tip. Most extensive growth was produced by those plants supplied with solution 56, and decreased growth was apparent in order of solution number, down to solution 50. It was noted, in caring for these plants lacking in nitrates, that rhizoids developed in abundance very early after starting the experiment. Those plants supplied with solutions lacking in phosphorus (solutions 29, 31, 34, 38, 43, 49, and 56) were dark green and produced an abundance of dark-colored rhizoids. The midribs were al-

most black, with a slight reddish hue. Except for those plants supplied with solution 56, which were much smaller than the others, all the plants in this group were practically alike in size and produced about as much growth as did the plants in other positions in the triangle, with the exception of those mentioned immediately below. Those plants supplied with solutions 30, 33, 37, 42, and 48 appeared at this time to have made much more growth than the other plants in the triangle. The only characteristic of this particular group which would account for their similarity in gross appearance at this time is the fact that all these solutions contained one-sixth of the PO_4 solution. This might indicate that maximum growth of *Marchantia* is associated with low concentration of phosphorus, which is at variance with the results attained with thalli grown from gemmae (**33**). A plant growing on solution 30 increased in area from 2.5 to 160 sq. cm. in 32 days. All plants in this experiment were harvested on May 27 (figs. 5, 6, 7, 8).

Discussion of experiments 8 and 9

The results of fresh and dry weight determinations of experiments 8 and 9 are recorded in tables 2 and 3. It is apparent that there were great differences in the amount of dry weight produced by various groups of plants supplied with the different solutions. On the basis of these dry weights, the rank of the various solutions was determined. Thus the solution with rank no. 1 produced the greatest amount of dry weight, and the solution with rank no. 28 produced the least, the other solutions ranking intermediately. The results of the counts on gemmae cup formation, antheridiophore and archegoniophore formation, and the area in square centimeters are recorded in tables 2 and 3.

The rate of accumulation of dry weight by *Marchantia* is markedly associated with the variations in nutrient solutions. In the cation triangle of both experiments 8 and 9, greatest growth was produced on the solutions in the central portion of the triangles. There was little change in the comparative rank of any given solution, regardless of the system used for calculation—whether on the basis of fresh weight or dry weight and of five or six plants. The responses of the antheridial plants are slightly different from those of the archegonial plants, but the correspondence is close considering the fact that the two experiments were carried out at different seasons of the year and under different conditions of intensity of daylight and average temperature.

On those solutions lacking in calcium the least growth was produced, and of this group the most growth was on solution 10, which contains about equal amounts of magnesium and potassium. As a group, it may be noted also that on those solutions lacking in potassium relatively little growth took place. A lack of magnesium did not affect the growth as much as the other absences, particularly in those solutions in which the calcium content was relatively high and the potassium content relatively low; for example, solutions 11 and 16.

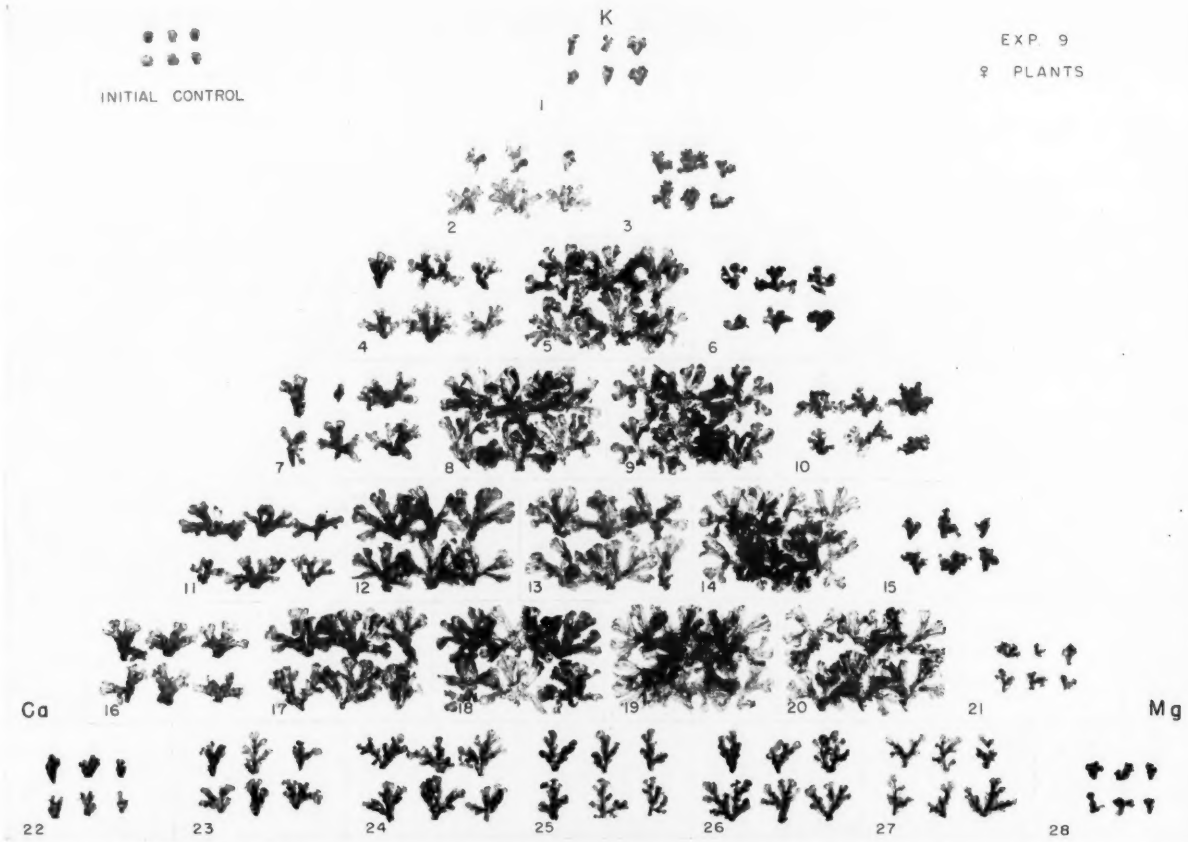

FIG. 5.—Cation triangle of experiment 9, May, 1940; archegonial plants on long photoperiod. Large amount of growth on some combinations necessitated overlapping the plants for photographing.

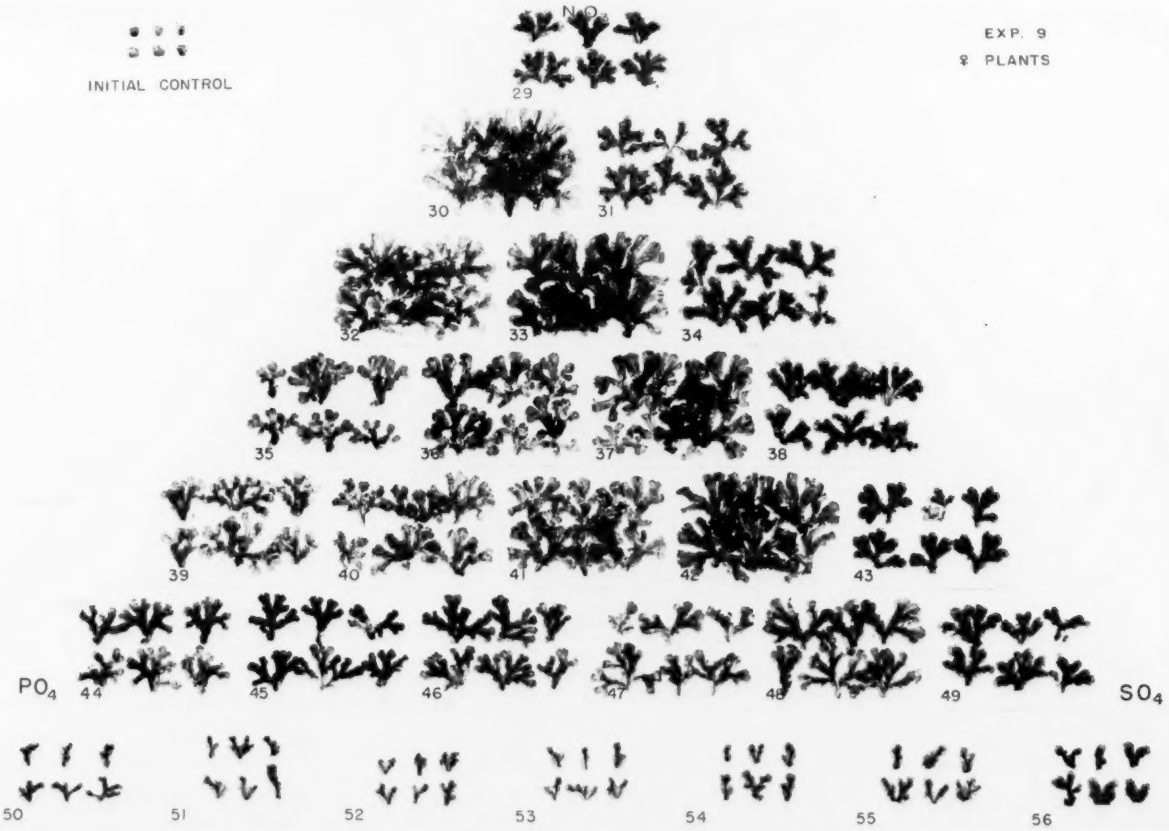

FIG. 6.—Anion triangle of experiment 9, May, 1940; archegonial plants on long photoperiod. Large amount of growth on some combinations necessitated overlapping the plants for photographing.

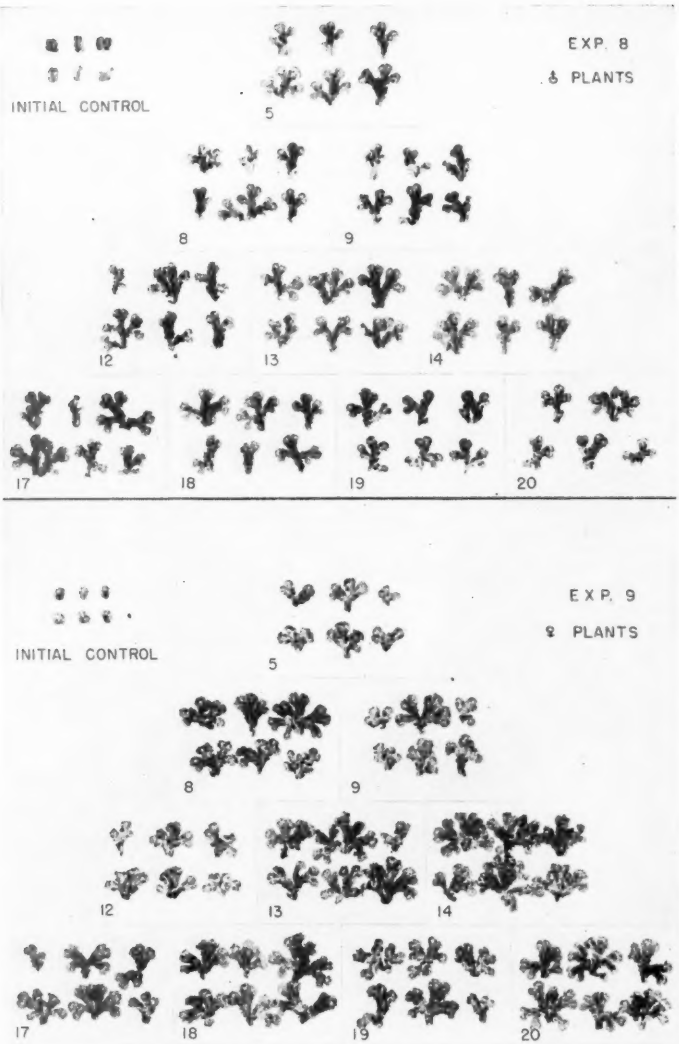

FIG. 7.—Cation triangles of experiments 8 and 9 on short photoperiod. Antheridial (above) and archegonial (below) plants grown April and May, 1940, respectively. Numbers correspond to same combinations as indicated in figs. 3 and 5. Total growth is much less than under long photoperiod (*cf.* figs. 3 and 5; tables 2 and 3).

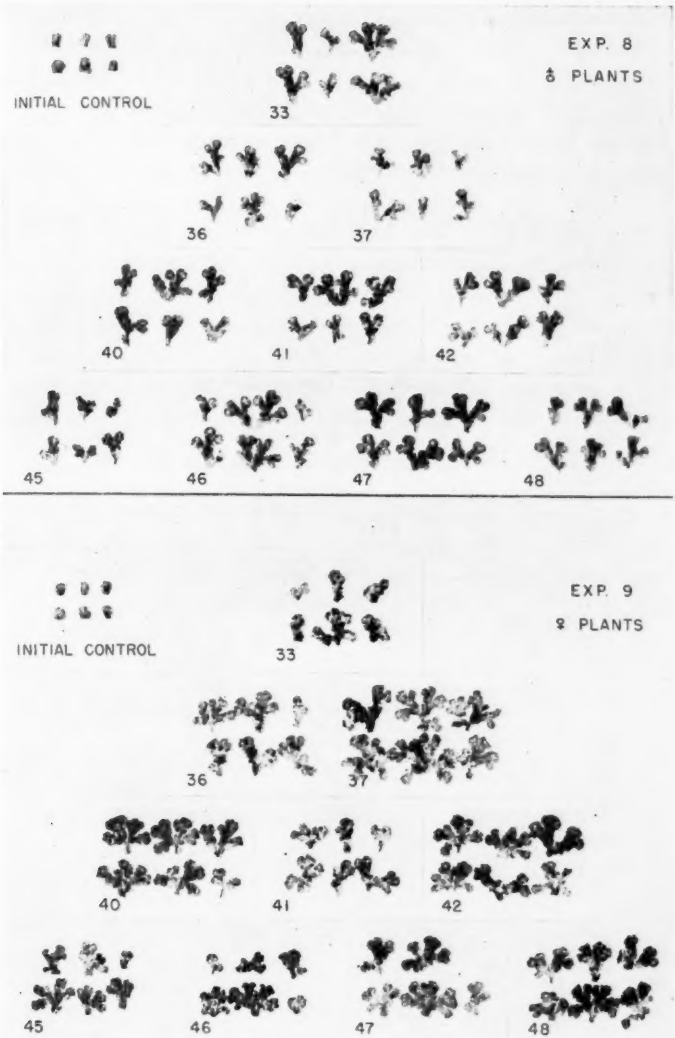

FIG. 8.—Anion triangles of experiments 8 and 9 on short photoperiod. Antheridial (above) and archegonial (below) plants grown April and May, 1940, respectively. Under short photoperiod the total growth is less than under long photoperiod.

In the anion triangles of both experiments there are several points of significance. In the first place, it is obvious that lack of nitrogen is accompanied by

TABLE 3

GROWTH OF MARCHANTIA POLYMORPHA ON NUTRIENT SOLUTIONS CONTAINING VARIOUS PROPORTIONS OF CATIONS OR ANIONS. ALL PLANTS GROWN ON SHORT PHOTOPERIOD

Position in triangle	Weight of 6 plants (gm.)				Area of 6 plants (sq. cm.)		No. of gemmae cups on 6 plants		No. of gametangiophores on 6 plants	
	Exp. 8		Exp. 9							
	Fresh	Dry	Fresh	Dry	Exp. 8	Exp. 9	Exp. 8	Exp. 9	Exp. 8 Antheridiophores	Exp. 9 Archegoniophores
	Cations K, Ca, and Mg varied proportionately on basis of sixths (cf. table 1) Anions present in constant relative proportion									
5	5.91	0.330	4.87	0.250	91	88	110	64	0	6
8	4.67	0.271	9.55	0.509	94	165	86	153	0	1
9	3.94	0.239	7.20	0.376	93	116	83	75	0	1
12	5.04	0.365	6.50	0.354	113	112	98	52	2	3
13	7.01	0.451	11.98	0.670	137	194	143	177	0	0
14	6.56	0.445	13.52	0.854	133	238	120	223	0	2
17	6.80	0.460	6.94	0.441	125	137	116	110	0	0
18	5.65	0.378	11.48	0.732	112	202	108	172	0	4
19	4.66	0.294	7.60	0.451	101	149	81	98	0	0
20	4.03*	0.274*	10.41	0.704	87*	201	90*	188	0*	2
Totals	.....	.....	.....	.....	1086	1602	1035	1312	2	19
	Anions NO_3, PO_4, and SO_4 varied proportionately on basis of sixths (cf. table 1) Cations present in constant relative proportion									
33	3.64	0.257	3.76	0.226	90	70	53	31	0	3
36	2.54	0.191	5.30	0.344	68	114	44	58	2	2
37	1.60	0.127	11.12	0.637	50	208	38	183	0	2
40	3.23	0.185	7.70	0.427	77	155	58	76	1	3
41	3.15	0.215	4.09	0.227	128	93	44	31	0	3
42	3.61	0.199	7.69	0.437	78	155	49	109	0	5
45	1.88	0.118	3.62	0.250	45	79	12	42	3	2
46	4.32	0.282	3.28	0.199	125	76	53	19	1	0
47	5.15	0.296	5.55*	0.372*	107	113*	75	76*	0	3*
48	3.08	0.211	6.94	0.418	75	138	57	109	4	3
Totals	.....	.....	.....	.....	843	1201	483	734	11	26

* Triangle position 20 in experiment 8 and triangle position 47 in experiment 9 possessed only 5 plants each at time of harvest. Had 6 plants been present it is assumed that all figures given would have been about one-fifth greater.

only slight growth, regardless of the content of phosphate or sulphate. It is also obvious that most growth occurred on those solutions low in phosphate and high in nitrate; that is, in solutions 30, 33, and 37. Considerable growth was also pro-

duced on solutions 42 and 48. Thus apparently a more marked effect was evident in relation to extremely small variations in the phosphate content than to any other single anion. The rate of accumulation of dry matter was apparently favored by low phosphorus content. Abundant phosphorus, particularly in those solutions where the nitrate content was low, was associated with decreased growth. Lack of sulphur resulted in decrease in the rate of growth on those solutions where the phosphate content was high but did not greatly affect the rate of growth on those solutions where the phosphate content was low (and the nitrate content high); for example, solutions 29 and 30.

More gemmae cups were produced on the antheridial than on the archegonial plants in both triangles (table 2). In the cation triangles more gemmae cups were produced on the solutions occupying a central position, particularly solutions 5 and 9, which were relatively low in calcium and magnesium and relatively high in potassium. Fewer gemmae cups were produced when there was an absence of potassium. In the anion triangles the production of gemmae cups was strikingly correlated with the phosphorus content of the solution. In both experiments more gemmae cups were produced on the solutions lacking in phosphorus than on any other, and the fewest gemmae cups were produced on the solutions lacking in nitrogen. There seemed little correlation between gemmae cup formation and sulphur content of the solution. Although in many combinations in both experiments numerous gemmae came to rest on the glass cloth and were permitted to remain relatively undisturbed for many days, none grew to form a mature thallus. Most gemmae disintegrated or were washed off the cloth when the solutions were changed.

The number of antheridiophores or archegoniophores produced on the various solutions was not clearly correlated with the concentration of any particular ion. In both experiments these structures were produced throughout both triangles. In the anion triangle of experiment 8 there seemed to be more antheridiophores on the solutions lacking phosphorus than on any other, although the correlation is not high. The determining factor in formation of antheridiophores and archegoniophores seems to be length of photoperiod rather than character of nutrient solution.

The total area produced by the plants growing on the various solutions was correlated rather closely with the accumulation of dry weight (tables 2, 3). Also, much greater growth was produced on certain solutions in the anion triangles of both experiments than was produced anywhere in the cation triangles. This is particularly true for solutions 30, 33, and 37, such solutions being relatively low in phosphorus. It may be that the phosphorus content of the cation triangles was slightly too high for maximum growth. If one wished to find a nutrient solution most favorable for the growth of this particular plant, consideration of the various

results recorded here would indicate that such a solution should have a relatively low phosphorus content and a relatively high nitrogen content. But to produce the maximum development of antheridiophores or archegoniophores, or of gemmae cups, it might be necessary to utilize in the nutrient solution an amount of phosphorus approximating 0.00015 mol per liter or less.

Comparative effects of long and short photoperiod on growth of Marchantia

As already noted, at the time experiments 8 and 9 were started, comparable plants were placed on solutions 5, 8, 9, 12, 13, 14, 17, 18, 19, and 20 and solutions 33, 36, 37, 40, 41, 42, 45, 46, 47, and 48, and exposed to conditions of short photoperiod. The area, fresh and dry weight accumulation, gemmae cup formation, and gametangiophore production are recorded in table 3.

The growth and accumulation of dry matter by these plants was much less than under conditions of long photoperiod. Practically no antheridiophores or archegoniophores were produced by any of the plants exposed to short photoperiod, and those which were produced were usually found in the older portions of the thalli and were probably already initiated at the time the experiment was started. The area of these plants paralleled closely their relative rate of accumulation of dry weight and was much less than comparable plants under conditions of long photoperiod. All plants exposed to short photoperiod produced an abundance of gemmae cups, and while the total number of cups produced by any particular set was in certain cases about the same as those produced by comparable plants exposed to long photoperiod, the number produced per unit area was always far greater. On most solutions the total number of gemmae cups produced by any particular group of six plants—growing on a specific nutrient solution and exposed to short photoperiod—far exceeded comparable plants exposed to long photoperiod. This is true in spite of the fact that the total area of these plants and their total dry weight were much less. On short photoperiod archegonial plants produced a greater total number of gemmae cups than did antheridial plants (table 3).

Internal anatomy

In experiments 8 and 9, one of the six thalli of each treatment was transferred to killing fluid immediately after the fresh weight of the lot was determined. After fixation, a representative thallus fork was selected for anatomical study. A piece of plant body, extending about 4 mm. from the apical notch toward the base of the plant, was trimmed so that its width approximated 2.5 mm. Immediately adjacent, a cross section of the thallus approximately 5 mm. wide was removed. Both plant fragments were imbedded in paraffin and sectioned at 7 μ. All sections were vertical; those through the apex longitudinal, and those through the more nearly mature part of the plant were transverse with respect to the axis of the thallus. The

longitudinal sections had in most instances meristematic, maturing and mature tissues. The cross sections had only relatively mature cells and tissues. Close microscopic examination of all preparations was made to determine whether any differences were apparent and whether such differences were possibly related to variations in nutrient supply and photoperiod.

Aside from such differences as width of thallus, coloration of scales and lower epidermis, development of rhizoids, and rapidity of dichotomy—characteristics which may be more readily determined by gross examination—very few striking anatomical differences were observable in the microscopic sections. Whenever the apical region of *Marchantia* was active mitotically, the number of cell divisions of the respective segments of the apical cells was about constant. Cell counts were made on both types of sections on nearly all the preserved plants. Although the thickness of the thallus varied considerably, the number of cells in a cross section was relatively constant. Counts were made vertically in a region twenty cells back of the apical cells in the longitudinal sections and in the midrib of the cross sections. Few thalli deviated from a cell count of 16 to 20; the greatest variation was from 11 to 26. Only the storage tissue was included in the count, to obviate the difficulties in recognizing cells in the photosynthetic areas in necrotic thalli. Plants growing on short photoperiod or on a solution deficient in one or two ions were thin vertically compared with plants growing on long photoperiod and a solution containing all the ions used in this experiment. Such thinner plants almost invariably possessed as many cells vertically as those thalli which appeared to be thick, the difference being in the size of the cells. Plants growing on solutions where necrosis occurred were thin in vertical distance and in the number of cells as counted vertically.

Anatomical effects of nutrient deficiencies

The results of anatomical studies are based on more than 1000 slides of serial sections. On long photoperiod some plants received solutions lacking in one or more cations or anions.

Potassium deficiency.—Plants grown in solutions very low in potassium possess no single anatomical alteration or condition attributable to such deficiency. Plants from solutions of the cation triangle numbering 22 to 28 inclusive (fig. 2) showed the customary anatomical development when a balance between calcium and magnesium was maintained. Necrosis was very marked in 22 (fig. 10*A*) and 28 (fig. 12*C*), the former lacking in calcium and the latter in magnesium (in addition to the absence of potassium), where large areas or nearly the entire thallus was nonliving (fig. 12*C*). Local necrosis was evident in 23 and 24 in female plants (grown in May). Plants on solutions 25, 26, and 27 were not necrotic except for the lower tip of the female plant in longitudinal section on solution 26. Anatomi-

cally the contrast between the plants on combination 27 and the nearly lifeless plants in position 28 is most striking. The former received one-sixth of the calcium solution and five-sixths of the magnesium solution, while the latter received the six-sixths magnesium solution only. In general, plants in the row lacking potassium stored more starch than plants in any other treatment, with the possible exception of the plants in the row lacking magnesium. Cell walls were not noticeably thick, but no plasmolysis occurred during fixation. Cells of the photosynthetic filaments were relatively large, distended, and possessed few chloroplasts. Pegged and smooth rhizoids were abundant.

CALCIUM DEFICIENCY.—The most striking anatomical changes were observed in relation to calcium deficiency. Apical growth in such plants ceases after a few days, and the usually green growing point becomes translucent and soon blackens. Large areas of such plants (1, 3, 6, 10, 15, 21, and 28) are necrotic, possess few rhizoids, and regenerate new thalli from the lower surface in the region of the midrib. Since regeneration was induced only in the plants lacking calcium, and since the development of new thalli occurs with such regularity, details will be discussed later in this paper. Death of the tips of the thallus was most rapid in combinations 21 and 28, which received relatively large percentages of magnesium. Regeneration occurred with greater regularity in 1, 3, and 6, becoming less frequent in 10, 15, and 21. Only one plant on combination 28 regenerated a thallus. Plants lacking in calcium generally possessed large upper epidermal and relatively few photosynthetic cells, which remained distended even when a layer of algae (mainly *Chlamydomonas*) completely covered the upper surface of the plant. These cells of the photosynthetic layer usually were devoid of chloroplasts.

MAGNESIUM DEFICIENCY.—Plants grown on combinations 1, 2, 4, 7, 11, 16, and 22 showed magnesium deficiency. The first and last combinations were also lacking in calcium and potassium, respectively. Plants in both treatments were necrotic and on combination 1 regenerated. Plants on the remaining solutions comprising the row in which magnesium was lacking were comparable with control plants in appearance, except that the mature upper epidermis and the mature photosynthetic layers were collapsed. Cells of these regions were well fixed when near the growing point, and immature. As seen in longitudinal sections there was a gradient of turgid cells to collapsed ones in the upper part of the thallus. Starch storage was pronounced in all treatments. The lower epidermis was thick walled and stained readily with safranin.

NITROGEN DEFICIENCY.—Characteristic growth responses were evident in the lowest row of the anion triangle. All plants were stunted, were light yellow in hue and deep red or purplish red underneath. This opaque red color was present in all the older scales and in the lower layers of the thallus, except the last 5 mm. of the tip, which was fairly translucent. The red color persisted after plants were pre-

served for nearly a year in F.A.A. solution (fig. 9*A*). The aquatic form or variety of *Marchantia* is described as possessing on the upper surface a median band pigmented with purple, associated with the absence of air chambers (**7**). Colored cell walls in liverworts have been ascribed to strong light combined with rather high temperature (**11**). MÖBIUS (**25**) reports that anthocyanin is present in the cell walls of *Marchantia* and persists for many years, even in a glycerin mount.

In longitudinal section (fig. 10*B*) the rather short zone of meristematic cells at the tip passes rather abruptly into the mature region of the thallus, which is characterized by large storage cells. Plants on a solution lacking in nitrogen were the first to develop rhizoids and were firmly attached to the glass cloth in a few days. No microscopic evidence was found of initiation of rhizoids nearer the tips. Dichotomy in plants lacking in nitrogen was infrequent, so that the narrowly elongated thalli were easily recognizable. Cross sections usually possess only one midrib (fig. 9*A*). Stored starch was evident in only a few of the plants, and all cell walls were thicker than in plants receiving all ions. The plant selected from combination 50 in experiment 9 was the only one showing a necrotic area among the anion plants. In a few scattered combinations, such as 10 and 16 in experiment 8 and 48 in experiment 9, under long photoperiod the lower epidermis and a few adjacent layers were relatively thick walled, but not as prominently so as in the nitrogen deficient plants.

PHOSPHORUS DEFICIENCY.—In these experiments plants grew well and to large size on solutions containing very small amounts of phosphorus. In fact there was considerable development, even though the solution contained no phosphorus—apparently a sufficient amount being present in the growing tips used for purposes of propagation at the beginning of the various experiments. The midribs of the thalli appeared black. Near harvest time it was obvious that the black coloration was a combination of the red lower surface and the dark green of the rest of the thallus. The older scales and the lower part of the thallus were red, much as in the nitrogen deficient plants, but possibly more intense. This color also persisted in F.A.A. solutions and in freehand sections was found to be limited to scales, rhizoids, lower epidermis, and at most the three cell layers nearest the lower epidermis. In the absence of fresh material it is presumed that the red color is the same for the plants when nitrogen or phosphorus is absent. The number of smooth rhizoids was very abundant (fig. 10*C*). In the midrib region pegged rhizoids often formed a bundle having a diameter which exceeded the width of the thallus. In the storage region of some plants lacking phosphorus (in experiment 9) cells were apparently plasmolyzed (fig. 10*C*).

SULPHUR DEFICIENCY.—Combinations 29, 30, 32, 35, 39, 44, and 50 contained no sulphur. Solutions 29 and 50 also lacked phosphorus and nitrogen, respectively, and the plants possessed the anatomical characteristics of these deficiencies.

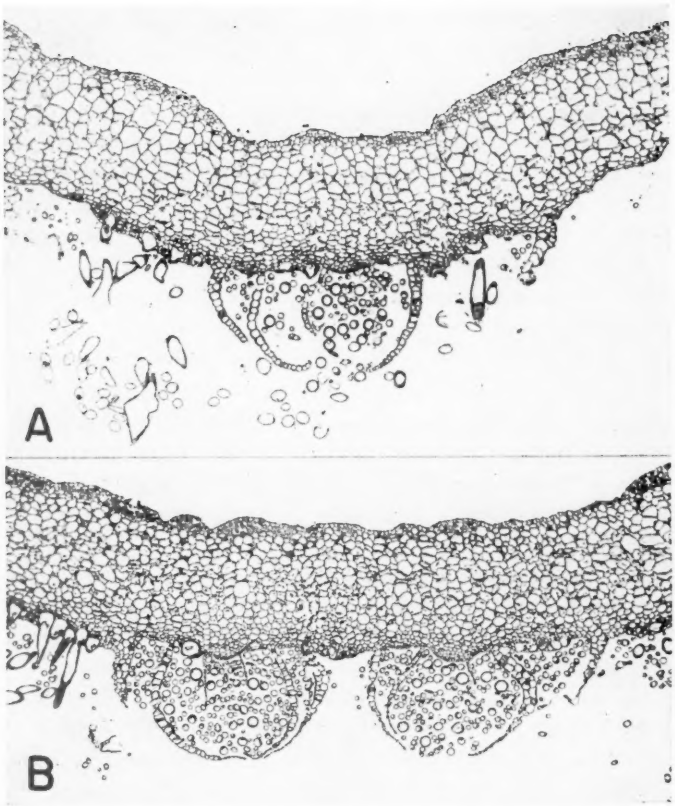

FIG. 9.—Cross sections of thalli in midrib region: *A*, nitrogen deficient plant on long photoperiod, experiment 8, on solution 55; cells of scales, rhizoids, lower epidermis, and adjacent layers are thick walled and colored red. *B*, initial control plant; darker staining cells below photosynthetic layer of thallus shown enlarged in fig. 14*A*.

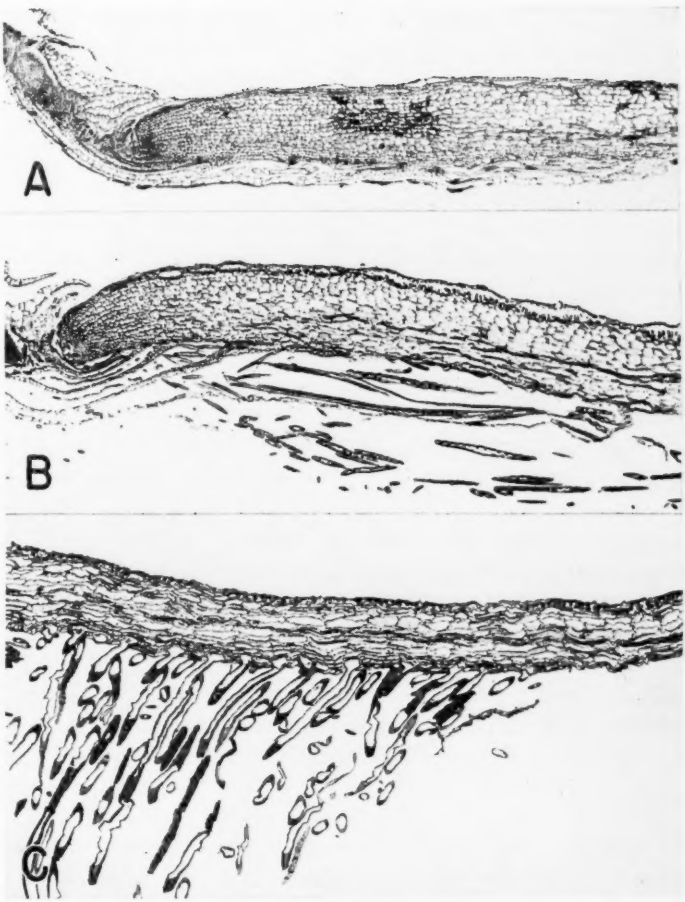

FIG. 10.—Vertical, longitudinal sections of thalli in midrib region: *A*, plant grown on combination 22 lacking potassium and magnesium but having relatively high concentration of calcium; experiment 8 on long photoperiod; several necrotic areas visible. *B*, plant grown on combination 54 lacking nitrates; experiment 9 on long photoperiod; mature region passes abruptly into less differentiated zone near tip. *C*, plant grown on combination 34 lacking phosphates; experiment 9 on long photoperiod; smooth rhizoids numerous.

Cross and longitudinal sections of plants on solutions 30, 32, 35, 39, and 44 resemble the sections of initial control plants and plants located in combinations in the center of the anion triangle in all respects. Apparently no sulphur deficiency existed, or such deficiency is not shown in anatomical details.

Effects of excess ions

Because of the nature of the experimental conditions, the greatest concentration of any ion was at the apices of the triangles (fig. 2). Combinations 1, 22, 28, 29, 50, and 56 contained the highest concentrations of the respective cations or anions, but each also was lacking in two ions. Limitation and alteration of growth and anatomical changes may not be ascribed to excess ions, unless adjacent combinations with a full complement of ions in some proportion also have similar characteristics. If comparable conditions are not present in the latter, it is reasonable to assign characteristics of growth and structure in the plants at the apices of the triangle to lack and not to excess of ions.

In the cation triangle, combinations with all ions present and with potassium, calcium, and magnesium in highest concentration were 5, 17, and 20, respectively. Plants grown on these combinations approximated the control plants in gross appearance and internal anatomy. Similarly, combinations 33, 45, and 48 contained all ions, and nitrates, phosphates, and sulphates were present in the largest amounts, respectively. Plants grown on these solutions also resembled the control or the original stock plants. Nutrient solutions of greater salt content, and consequently greater osmotic concentration, could be employed. Any appreciable anatomical change in plants grown on the combinations just discussed could be attributed to excesses if similar conditions were not found in plants receiving a smaller quantity of a particular ion. No anatomical effects of excess ions were observed in microscopic preparations of plants of experiments 8 and 9.

Interaction of ions

In combination 22, which was high in relative concentration of calcium ions, necrotic areas were present throughout the thallus (fig. 10*A*). Similar but less marked necrosis was also present in plants grown on solutions 23 and 24. These also were on relatively high calcium solutions, five-sixths and four-sixths, respectively. Plants on combination 26 were nearly devoid of necrosis, and thalli on 25 and 27 lacked necrotic areas entirely. Plants on solution 28 were almost wholly necrotic (fig. 12*C*). Since all combinations (22 to 28) lacked potassium, such diversity of anatomical expression cannot be ascribed to its absence alone. These combinations varied conversely in calcium and magnesium content, and it is likely that high calcium supply accompanied by low magnesium supply—together with lack of potassium—favored necrosis. High magnesium supply accompanied with

varied calcium supply (one-sixth to three-sixths) and absence of potassium favored the usual anatomical development in *Marchantia*.

In the row of nutrient combinations lacking calcium (1, 3, 6, 10, 15, 21, and 28), low magnesium supply and attendant high potassium supply favored thallus regeneration, but a nutrient supply with converse proportions of these cations retarded the formation of adventitious thalli.

No interrelationships of anatomical effect on plants were noted with the cation magnesium and the other cations, and none of the anions interacted to produce an anatomical change great enough to be observed.

Anatomy of plants supplied with all nutrient ions in various proportions

Plants on combinations 5, 8, 9, 12, 13, 14, 17, 18, 19, and 20 in experiments 8 and 9 are very similar anatomically. Such plants grew large in area, elongated rapidly (at times even elongating their gemmae cups), and possessed rather brittle thalli. As a rule the upper epidermis was shrunken in microscopic preparations, and the photosynthetic filaments were partially collapsed. Walls of cells in the central portion of the storage region were so thin that many cells collapsed, forming a strandlike zone running longitudinally through the thallus. Scales are formed at the apex of the plant but are shrunken and inconspicuous in the mature region. Both types of rhizoids develop as in control plants.

In the anion triangle, combinations receiving all ions in various proportions were 33, 36, 37, 40, 41, 42, 45, 46, 47, and 48. Again the anatomy of plants in experiments 8 and 9 is very similar. As a rule the cells and tissues in these plants were comparable with plants supplied with all ions in the cation triangle. No single characteristic nor any obvious set of criteria was found on which to separate plants in these twenty positions from one another. Antheridial plants in experiment 8 and archegonial plants in experiment 9 reacted similarly.

Anatomical effects of short photoperiod

Anatomically the effect of short photoperiod on plants in both triangles, in both experiments, was identical. Only the twenty combinations just mentioned were used on short photoperiod. All plants appeared thinner in vertical section than plants in similar positions on long photoperiod. Cell counts in cross section in the storage region averaged 16 to 20, a range that duplicates the cell count in plants on long photoperiod. In the storage region of these plants the upper 5–7 cell layers averaged 40–50 × 110–125 μ per cell. Cells in the next 4–6 layers averaged 20 × 75–115 μ. Cells nearest the lower epidermis and several layers above averaged 10–20 × 40 μ. The long axis of all cells was parallel to the axis of the thallus. The uppermost layer of storage cells averaged larger in size than comparable cells

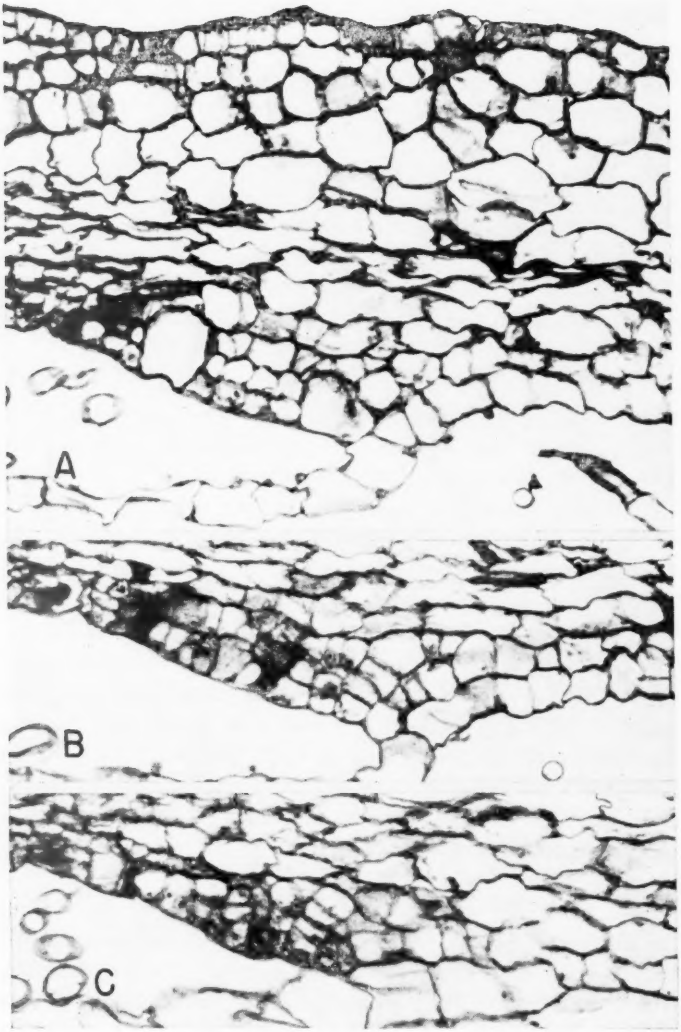

FIG. 11.—Vertical, longitudinal sections of thalli, experiment 8, combination 3, on long photoperiod: *A*, at left, bases of two rhizoids; *B*, similar section. Epidermal and subepidermal cells between bases of rhizoids have become meristematic; *C*, similar section showing more advanced meristematic condition, beginning of regeneration of new thallus.

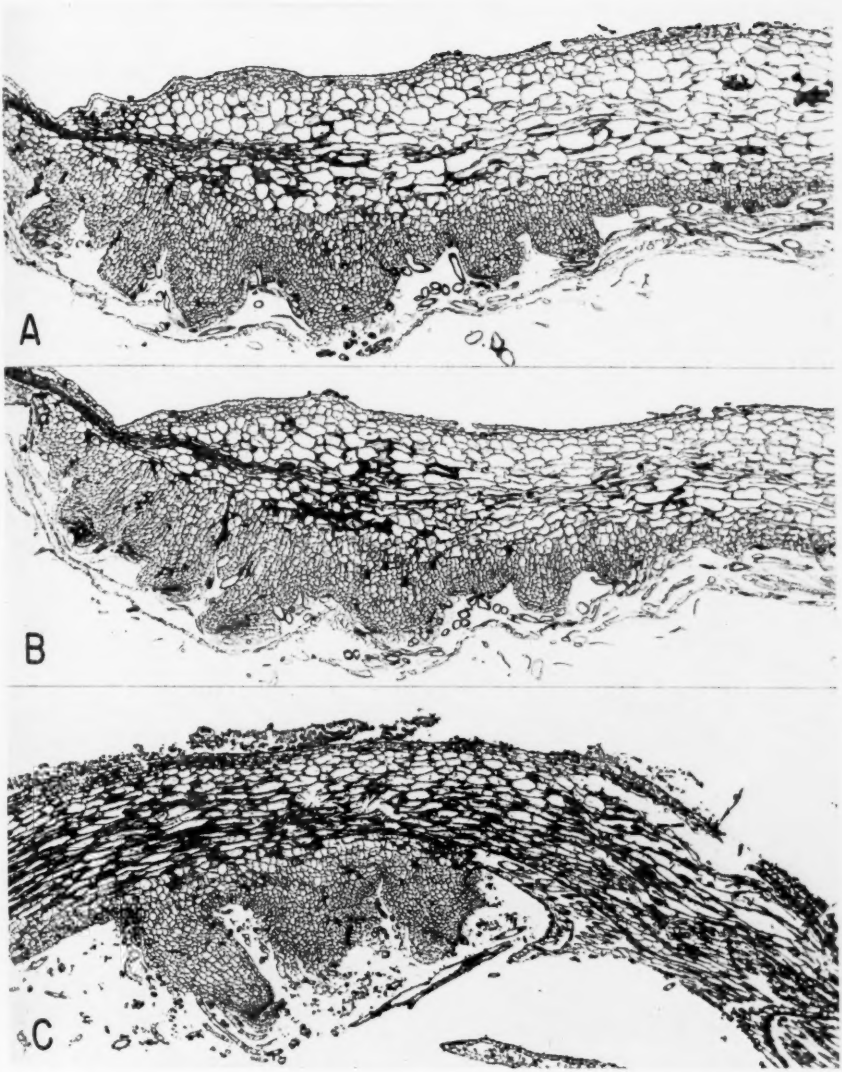

FIG. 12.—Similar to fig. 11, showing progressive stages at *A*, *B*, and *C* of regeneration of thalli

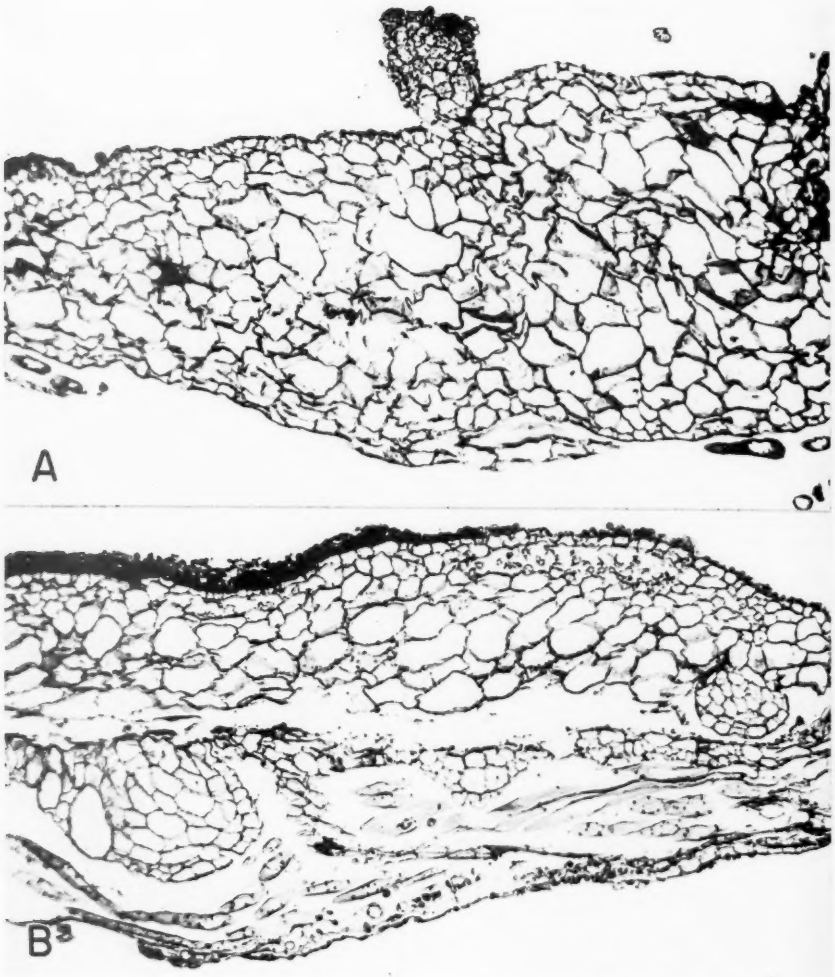

FIG. 13.—*A*, regeneration of young thallus at upper surface of old thallus; *B*, regeneration of thalli from lower surface and from storage cells of old thallus.

in any of the other treatments. The smaller dimensions of the other layers in the storage region, however, reduced the total thickness of the thallus. Gemmae cups in many stages of development were found in nearly all plants sectioned.

Lack of calcium and regeneration of thallus

All combinations in both experiments in which calcium was lacking (1, 3, 6, 10, 15, 21, and 28) regenerated new thalli from the underside of the midrib region. Although such regenerated thalli never became very large, they were visible to the unaided eye. On solutions 1, 3, and 6, numerous thalli were formed; in some cases a regenerated thallus in turn regenerated another one. Fewer thalli were formed on combinations 10, 15, 21, and 28; on the latter, only one of the sectioned plants in experiment 9 possessed a regenerated thallus. Seven-μ sections of *Marchantia* plants on these combinations presented a series of developmental stages of new thalli (figs. 11–13).

Cells of the lower epidermis on either side of the midrib region, in interrupted longitudinal rows, may differentiate into large, dark-staining cells characteristic of rhizoid initials. These elongate as smooth rhizoids when the apical region has outdistanced them by about twenty cells, although some initials remain dormant for a time. When mature (fig. 9*A*, *B*), such rhizoids are smooth walled, grow vertically downward, and attach to the substrate (**7, 13**). The base of a rhizoid is large, and cells surrounding it usually undergo nuclear and cell divisions but do not enlarge appreciably. The rhizoid base has the appearance of being sunken two or three cell layers into the lower side of the thallus (fig. 11*A*). In plants grown on solutions lacking calcium, these small epidermal and subepidermal cells surrounding the base of the rhizoid become meristematic (fig. 11*B*). A small group of cells is formed which protrudes from the lower level of the epidermis (fig. 11*C*). Initiation of such meristematic groups occurs in basipetal succession (fig. 12*A*). The meristematic group of cells soon differentiates a mucilage hair (slime papilla), accompanied by a small, acropetally placed depression. In this depression, apical cells are differentiated. Groups of such growing points often occur simultaneously to form a longitudinal row of thalli (fig. 12*A*, *B*, *C*). In median longitudinal section young thalli have the same structure as adult plants, except that meristematic activity is limited to the apical cells and one subepidermal row (fig. 12*B*, *C*). The tips of all regenerated thalli are directed toward the tip of the parent plant and may emerge from beneath it. In all plants regenerating ventrally, only the lower epidermis—and at most about five cell layers of the lower part of the thallus—are involved in the production of new thalli.

Two types of exceptions to the method of regeneration just described are shown in figure 13. The uppermost thallus has produced an adventitious bud from the upper epidermis (combination 10, experiment 8). Only one other such example

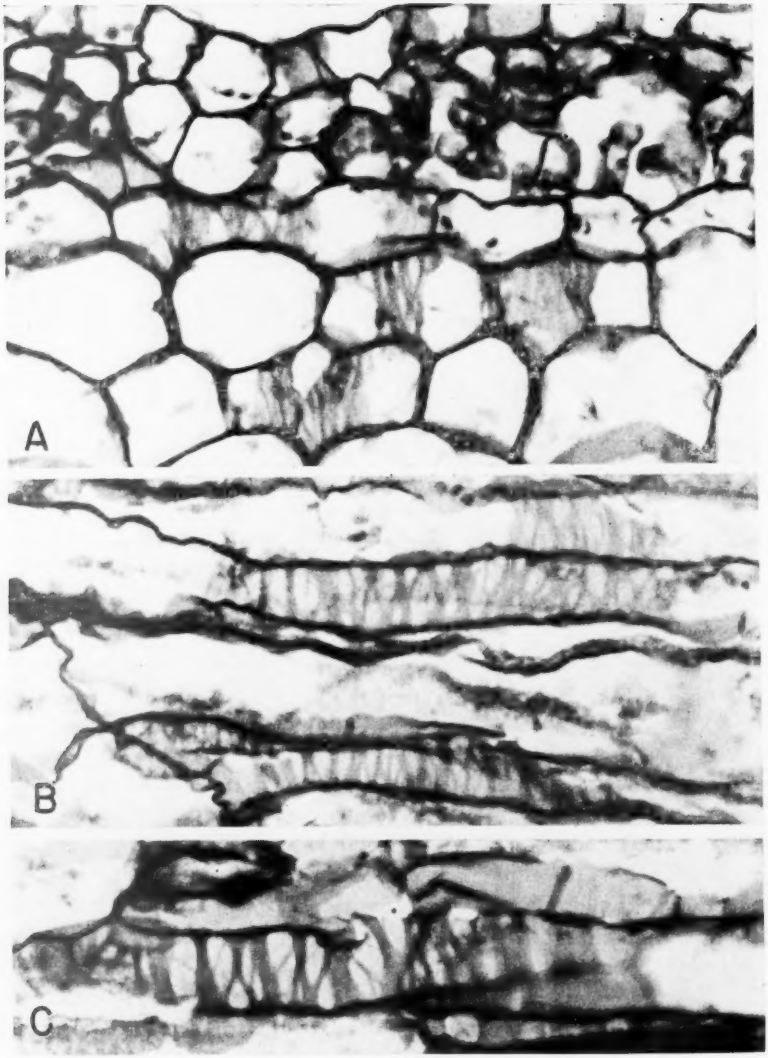

FIG. 14.—Cells of storage region showing wall thickenings as commonly found in all cultures: *A*, transverse section; *B*, *C*, longitudinal sections.

was found (combination 3, experiment 9). In this variation of regeneration only a few upper layers of cells were involved in the production of the new thallus. Because of the absence of air chambers (fig. 13*A*), it is known that the bud was formed in the midrib region which in this species commonly lacks air chambers.

The second type of exception to the usual method of regeneration was found in one thallus only (combination 3, experiment 9). Necrosis had occurred in a longitudinal layer of the storage tissue, a few cells above the lower epidermis (fig. 13*B*). A part of this necrotic strand disintegrated, leaving an air space. Storage cells forming the roof of this space became active and formed a small, internal bud. The parent thallus also produced a bud from the lower epidermis in the usual manner (fig. 13*B*).

KLEIN (**20**) reports that *Marchantia polymorpha* growing naturally in Hungary regenerated from the furrows of the archegoniophores. Several investigators have shown that in thallus cuttings of thallose liverworts regeneration proceeds from the ventral surface of the apical end (**2, 6, 21, 36**). VÖCHTING (**36**) illustrates the younger stages of regeneration in the furrow of the archegoniophore of *Marchantia* following injury. The absence of adventitious bud formation on all nutrient combinations containing only the smallest fraction of the calcium solution indicates that the absence of calcium or the interaction of factors in the absence of calcium may act to stimulate certain ventral cells to become active.

STORAGE CELLS WITH WALL THICKENINGS AND PITS

On every nutrient combination and under both photoperiods, *Marchantia* plants possessed elongated cells in the storage region which were thickened on the inside face of their walls. At times these thickenings were very faint and resembled protoplasmic strands. End walls of storage cells of the initial controls possessed scalariform thickenings (fig. 14*A*). Such thickenings varied in width (fig. 14*B*, *C*). These cells were found either near the photosynthetic tissues (fig. 14*A*), in the middle of the thallus, or very near the lower epidermis (especially on solution 56). They were most prominent in longitudinal sections near the midrib and appear to have been living cells at the time of fixation, since starch grains were present in several such cells (combinations 16 and 11 in experiments 8 and 9, respectively). In necrotic thalli such as those on combinations 10, 15, 21, and 28, these thick-walled cells stained deeply with safranin, which is characteristic of nearly all the nonliving cells. Cells from plants lacking in calcium possessed thickenings which covered most of the wall, leaving only large oval pits.

Storage cells with walls thickened in various ways have been repeatedly mentioned by bryologists for *Marchantia* (**3, 7, 12, 18, 26, 35, 37**), *Conocephalum* (*Fegatella*) (**2, 4, 27**), *Lunularia* (**32**), *Monoclea* (**30**), *Bryum* and *Mnium* (**15**), and members of the Dilaenaceae such as *Symphyogyna* (**22**). Isolated drawings of such

cells are presented by BOLLETER (2), CAVERS (4), and SCHIFFNER (32) for *Conocephalum* and *Lunularia*, respectively. GOLENKIN (14) figures mycorrhizal hyphae inhabiting red-violet storage cells in *Preissia*, *Marchantia palmata* and *M. paleacea*, all of which are said to be pitted in age. The tracheids found by HOLLOWAY (19) in the gametophyte of *Psilotum* probably belong to a category different from those described in the accompanying figures.

General discussion

While the foregoing results are based upon relatively few plants on each combination of nutrients, some of the data are striking and clear cut. Growth of *Marchantia* is greater on nutrient solutions with a low osmotic concentration than on those of a relatively high one. The effect of length of photoperiod is also marked; vegetative extension, gametangiophore production, and vegetative activity are greater under long photoperiod than short photoperiod, although under the latter condition formation of gemmae cups is increased. This seems true regardless of the nutrient solution upon which the plants are grown. In our experience it has also been found that the particular strains of *M. polymorpha* used will grow very rapidly, even under conditions of relatively high light intensity and relatively high greenhouse temperatures, dependent upon the composition of the nutrient solution supplied. It has been found repeatedly in different experiments that plants growing on the various nutrient solutions produced much greater growth than did comparable plants growing on garden loam soil, even though the plants on such soil were abundantly supplied with water. On some of the nutrient combinations the plants grew and gained more dry weight than did plants observed under field conditions at comparable seasons of the year. The only plants which have been observed growing as rapidly and as large in natural habitats were found on areas from which vegetation had recently been burned or on the charcoal and ash residues of burned trash piles.

The marked effects resulting from variations in the proportions of the major cations and anions indicate clearly that *Marchantia* produces much greater growth on nutrient solutions relatively low in phosphorus content, regardless of great variation in sulphate and nitrate content. It is likely that with certain combinations even greater growth may be produced. If, for example, larger triangles were used, so that the variations were not so great from one combination to another, and if the number of plants grown on each particular solution were greater, a more rigorous statistical analysis of the results could be made. It is highly desirable to establish cation triangles in which much lower concentrations of phosphorus are present, and it would be equally desirable to test higher concentrations of nitrate. Under such range of combinations in the cation triangle, it might well be that small differences in the content of potassium, calcium, or magnesium would show

even more striking effects than were obtained in the present experiments. The method used is applicable to the general study of problems of mineral nutrition.

Summary

1. *Marchantia* grows readily on glass cloth in an open moist chamber when supplied with an appropriate nutrient solution. A method for growing it under controlled conditions of nutrient supply and photoperiod is described. A male and a female strain of *Marchantia polymorpha* were employed, each strain propagated vegetatively from a stock grown from a single plant.

2. A range of fifty-six nutrient solutions, each having an osmotic concentration of approximately 0.285 atmospheres, was used. The cations involved were K, Ca, and Mg, and the anions NO_3, PO_4, and SO_4.

3. Cultures containing the cations K and Mg but without Ca all regenerate new thalli, formed in the main from adventitious buds arising from cells on the ventral side of the thallus at the midrib region. In cultures lacking NO_3 and PO_4 the ventral layers of cells develop a red-violet color in the walls. The absence of Mg and SO_4 does not result in so striking a response as lack of the other ions. Phosphate is required in very small quantities. Up to the limits of the quantities supplied, plants make greater growth with increasing amounts of NO_3, provided all other ions are present.

4. Some of the plants were grown on long photoperiod, 18 hours of light out of each 24-hour cycle, and on short photoperiod, 9 hours of light out of each 24. The differences between the lots of plants grown on the two photoperiods were both quantitative and qualitative. Irrespective of the range of nutrient supply, plants on long photoperiod were larger and had greater dry weight than similar plants on comparable solutions on short photoperiod. Irrespective of the range of nutrient supply, plants on short photoperiod produced a greater total number of gemmae cups but on long photoperiod a greater number of gametangiophores than comparable plants on short photoperiod.

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CHEMICAL AND HISTOLOGICAL RESPONSES OF BEAN PLANTS GROWN AT DIFFERENT LEVELS OF NUTRITION TO INDOLEACETIC ACID[1]

ORA SMITH, L. B. NASH, AND G. E. DAVIS

(WITH ELEVEN FIGURES)

Introduction

Considerable histological work has recently been published on the responses of various plants, including the bean (**3, 4, 5, 6**), to applications of indoleacetic acid and other growth substances. Other investigations (**1, 7, 8, 9, 10**) have shown that application of indoleacetic acid to cuttings or decapitated plants affects their metabolism and rate of transfer of carbohydrate and nitrogenous compounds. The writers, however, know of no investigation wherein the histological studies and chemical analyses were made on the same material grown under the same conditions at the same time. It has not yet been shown whether roots are formed after translocation of certain materials, probably as a result of it, or whether the transfer of carbohydrate and nitrogenous materials occurs after root primordia have been stimulated more directly by application of the growth substance. The present paper relates certain histological changes to chemical changes of the same lots of plants grown under the same conditions.

There is little published work (**3**) on the effects of various levels of nutrition of the plants on their response to the growth substance application. Obviously differences in nutrition may greatly alter this response. Again, most studies have involved plants from which the roots or the terminal growing point were removed before treatment. Only the cotyledons were removed from the seedlings used in these studies.

Material and methods

CHEMICAL STUDIES.—Seedlings of kidney bean, *Phaseolus vulgaris*, variety Pencil Pod Black Wax, were grown under two levels of nutrition. One level secured seedlings high in nitrogen and low in carbohydrates, and the other level secured seedlings low in nitrogen and high in carbohydrates. On July 21, about 2500 seedlings of each level of nutrition were started in washed quartz sand. Flats 4×3 feet were used, and all seedlings were grown in the greenhouse.

The high nitrogen, low carbohydrate seedlings were grown as follows: complete nutrient solution was added each day, and the sand was kept sufficiently moist

[1] Paper no. 213, Department of Vegetable Crops, Cornell University, Ithaca, New York. Indoleacetic acid supplied by Merck & Co. and Eastman Kodak Co.

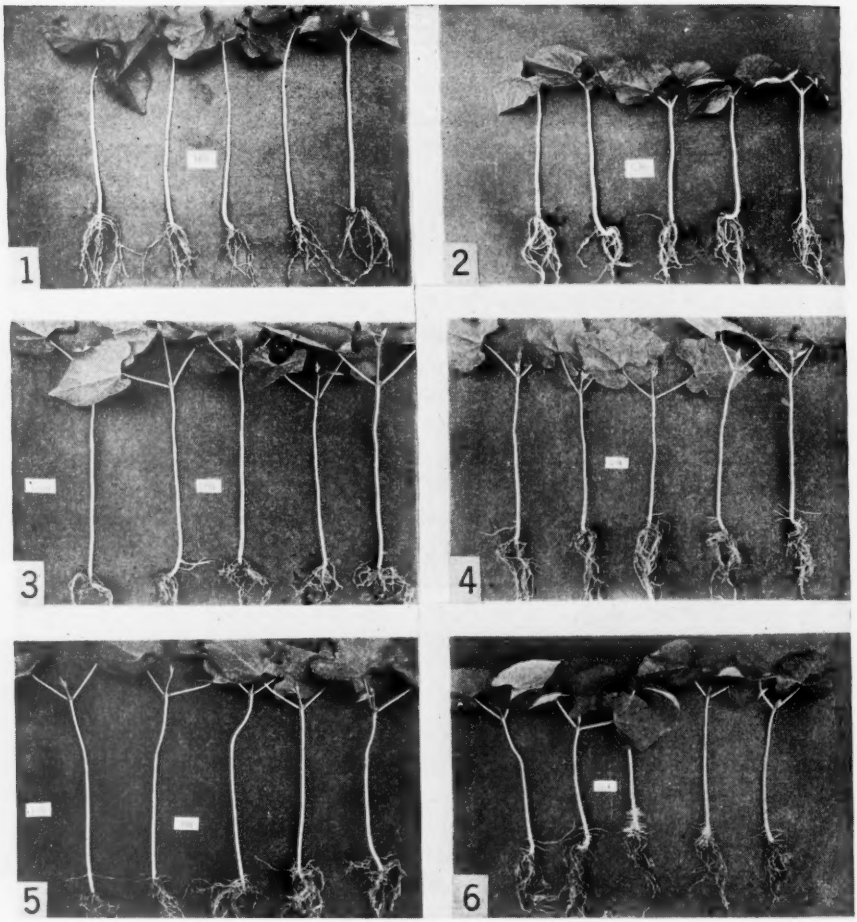
for continued growth. During the 2 days previous to treatment the seedlings were shaded by brown wrapping paper placed about 6 inches above them. The low nitrogen, high carbohydrate seedlings were grown as follows: nutrient solution containing nitrogen was added in sufficient quantity at the time of planting to sprout the beans, but after the first addition only enough nitrogen-free solution was added each day to keep the seedlings from wilting. The sand was kept dry. As a result growth was checked and carbohydrates accumulated. These seedlings were also given 4 hours of artificial illumination, each of the two nights previous to treatment. Two 100-watt bulbs were used above each flat.

As was expected, the high nitrogen, low carbohydrate seedlings were tall, spindly, and soft as compared with the shorter, stockier, and hardier low nitrogen, high carbohydrate seedlings (figs. 1, 2).

Eight days after planting, when the second internode was about 1 cm. in length, the seedlings were prepared for treatment. The cotyledons were removed and all seedlings were taken from the flats. The seedlings, carefully selected for uniformity, were treated by standing in bunches of about 100 in a 0.01 per cent aqueous solution of indoleacetic acid or in tap water, placed so that all the roots and about 1 inch of the lower hypocotyls were submerged. The indoleacetic acid was first dissolved in a small quantity of ethyl alcohol. Duration of the treatment was 4 hours. After treatment, the seedlings were washed with tap water and transplanted into washed quartz sand in large greenhouse flats. All seedlings were then watered with complete nutrient solution and otherwise treated the same. For the first 2 days after treatment, the seedlings were kept in the headhouse and then removed to benches in the greenhouse, where the light had been previously reduced by a coat of whitewash on the glass. The temperature ranged from 70° to 100° F. The benches and floor of the house were kept moist enough so that the plants did not wilt badly during the middle of the day.

Samples of 100 representative seedlings from each of the treatments were taken between 9 A.M. and 10 A.M. and sectioned into roots, lower and upper half of hypocotyl, first internode, and leaves. The leaf sample included the petioles. The dry weight of these sections was determined by placing in a forced draft oven at 85° C. for 48 hours and weighing. Total nitrogen to include nitrates and total sugars were determined by methods given by the A.O.A.C. (2). Alcohol-insoluble acid-hydrolyzable carbohydrates were determined on the residue from the sugar extraction and were calculated as starch.

HISTOLOGICAL STUDIES.—Samples for histological observation were taken from about ½ inch of the lower end of the hypocotyl, since this is the region where new roots are expected to appear. These sections were fixed in a modified chromo-acetic acid mixture, imbedded in paraffin, and cut 15–20 μ in thickness. Both longitudinal and cross sections of the hypocotyl were made in order to observe as



FIGS. 1–6.—Fig. 1, high nitrogen, low carbohydrate seedlings at time of treatment with indoleacetic acid solution 8 days after planting. Fig. 2, low nitrogen, high carbohydrate seedlings 8 days after planting. Figs. 3–6, plants 6 days after experiment was started: 3, high nitrogen, low carbohydrate seedlings not treated. 4, low nitrogen, high carbohydrate seedlings not treated. 5, high nitrogen, low carbohydrate seedlings treated with 0.01 per cent acid. 6, low nitrogen, high carbohydrate seedlings, treated.

much of the tissue as possible. The tissue was stained with safranin and counterstained with crystal violet in clove oil.

The preceding samples were taken from all treatments at 12-hour intervals up to 48 hours; thereafter at 72, 96, 144, and 192 hours and 8, 11, and 14 days.

Experimental results

Growth responses

Treatment of seedlings with indoleacetic acid influenced subsequent growth at both levels of nutrition. Treatment increased the production of roots, suppressed elongation of the first internode, and delayed development of the second internode. At both levels of nutrition the suppression of top growth in the treated seedlings was about equally marked; but great difference was found in the time of production of new roots (table 1, figs. 3–6).

TABLE 1

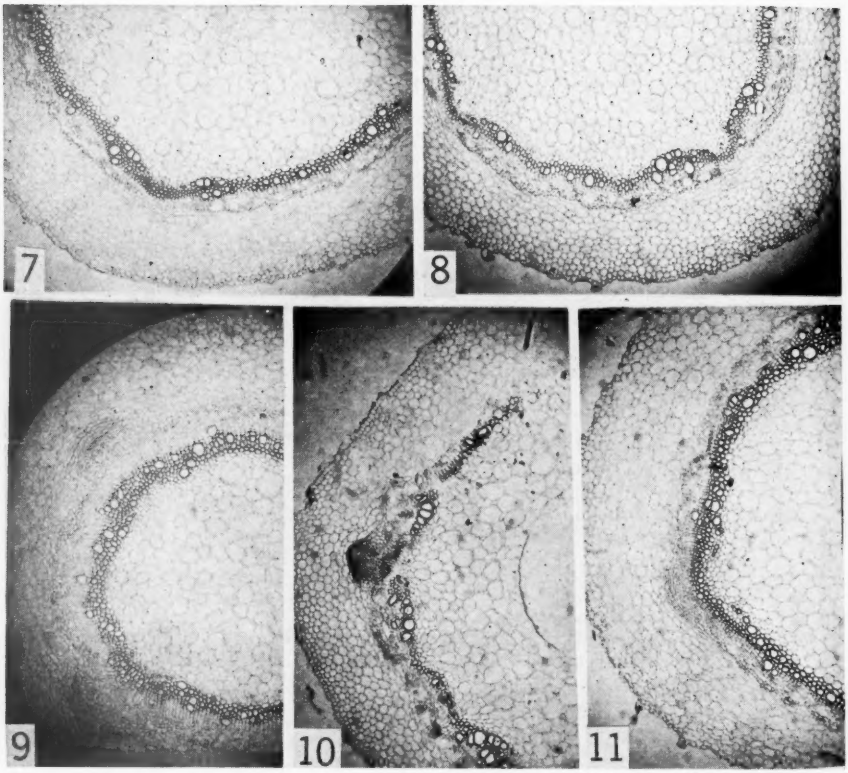
Time of appearance of root primordia and visible roots from hypocotyl

Treatment	Appearance of first root primordia in hypocotyl	Appearance of new roots outside hypocotyl
1. Low nitrogen, high carbohydrate, treated	36 hours	48 hours
2. Low nitrogen, high carbohydrate, not treated	72 hours	96 hours
3. High nitrogen, low carbohydrate, treated	144 hours	192 hours
4. High nitrogen, low carbohydrate, not treated	None after 268 hours	None after 268 hours

Cross sections of the lower hypocotyls of untreated plants at the start of the experiment are shown in figures 7 and 8.

Under conditions of low nitrogen, high carbohydrates, and treated with indoleacetic acid, root primordia were observed scattered throughout the hypocotyl 36 hours after treatment. These primordia apparently originate from the phloem tissue, above the intervascular area, and are frequently found on the same level at four diagonally opposite points on the hypocotyl (fig. 9). In plants of this lot new primordia continued to be laid down through the sixth day after treatment. These new primordia could be observed together with the more developed secondary roots which had been initiated earlier. Apparently new root initiation stopped soon after the sixth day, as none was observed in the samples taken later. In plants low in nitrogen and high in carbohydrates, new root primordia were found at 72 hours without treatment with indoleacetic acid, but not in the same abundance as in those treated with the acid (fig. 10). A few primordia were found again at 6 days and none thereafter.

Treating the high nitrogen, low carbohydrate plants with indoleacetic acid stimulated root primordia to appear by the sixth day after treatment (fig. 11).



FIGS. 7–11.—Figs. 7, 8, cross sections of lower hypocotyls of seedlings at beginning of experiment: 7, low nitrogen, high carbohydrate. 8, high nitrogen, low carbohydrate. Figs. 9–11, cross sections of lower hypocotyls showing root initials: 9, treated, low nitrogen, high carbohydrate seedlings, 36 hours after treatment. 10, low nitrogen, high carbohydrate seedlings, untreated, 72 hours after treatment. 11, treated, high nitrogen, low carbohydrate seedlings, 6 days after treatment.

Under the same conditions but without treatment no primordia could be found in any of the samples through the eighteenth day after treatment, when the experiment was terminated.

Treated plants produced more roots than did the untreated plants. After treatment, however, roots appeared much sooner and in greater numbers in the low nitrogen, high carbohydrate plants than in the high nitrogen, low carbohydrate plants. This was true for induced roots on the lower hypocotyl as well as for new secondary roots on the pre-existing root systems.

Conditions of low nitrogen or high carbohydrates are conducive to the production of new roots on the lower hypocotyl without indoleacetic acid treatment, although roots appear in not nearly the same quantity nor so early as when treated. Since no root primordia could be observed under conditions of high nitrogen, low carbohydrates, without treatment, it follows that in these experiments the formation of new root primordia was largely influenced by the carbohydrate reserves in the plant.

Influence of indoleacetic acid on distribution of dry weight

The seedlings were kept for the first 2 days after treatment in the headhouse, where the light intensity was low and the temperature relatively high. The greenhouse to which the seedlings were transferred had previously been shaded by a coat of whitewash on the glass. Consequently there was little actual gain in dry weight the first few days after treatment.

The data of table 2 show that there were differences in distribution of materials in the seedlings. As already pointed out, treating the low nitrogen, high carbohydrate seedlings with indoleacetic acid markedly increased the number of roots, and table 2 shows also an increase in the dry weight of the roots. However, treatment of the high nitrogen, low carbohydrate seedlings resulted in little or no consistent increase in dry weight of the roots above those untreated. The dry weight of the lower hypocotyls increased in all treatments for the duration of the experiment, but after 4 days the dry weights of lower hypocotyls of the untreated seedlings were greater with one exception than the treated. The probable reason for this greater increase after the first 4 days is that material in this region was not translocated into the roots as fast as from the treated lower hypocotyls. In the treated, low nitrogen, high carbohydrate seedlings the plants had a lower dry weight of the first internodes at every sampling and also of the upper hypocotyls after the first sampling than the untreated ones.

In the high nitrogen, low carbohydrate seedlings it was not until 8 days or more after treatment that the dry weights of the upper hypocotyls and first internodes

of treated seedlings were appreciably and consistently lower than of the untreated seedlings.

This would indicate that the treated, high nitrogen, low carbohydrate seedlings were less responsive to changes in the distribution of dry weight than were the treated, low nitrogen, high carbohydrate seedlings.

TABLE 2

DRY WEIGHT (IN GRAMS) OF 100 KIDNEY BEAN SEEDLINGS TREATED WITH WATER AND WITH 0.01 PER CENT AQUEOUS SOLUTION OF INDOLEACETIC ACID

Days after treatment	Roots		Lower hypocotyls		Upper hypocotyls		First internodes		Leaves	
	Water	Acid	Water	Acid	Water	Acid	Water	Acid	Water	Acid
	High nitrogen, low carbohydrate									
0	2.028	.....	2.024	.....	1.521	.....	1.155	.....	6.782	.....
2	2.173	2.296	2.592	2.184	1.983	1.641	1.437	1.297	7.693	7.255
4	2.200	2.221	2.448	2.373	1.882	1.923	1.566	1.528	7.988	9.399
6	1.992	2.613	2.596	2.634	1.956	2.290	1.549	1.691	8.653	10.249
8	3.234	3.672	2.829	2.562	2.192	2.050	1.732	1.807	9.315	10.877
11	2.889	2.939	3.523	3.029	2.775	2.204	2.333	2.056	11.926	12.441
14	5.079	4.030	3.773	3.251	2.948	2.478	2.537	2.257	12.644	12.301
	Low nitrogen, high carbohydrate									
0	3.521	.....	1.845	.....	1.725	.....	0.866	.....	7.126	.....
2	1.983	2.813	1.980	2.141	1.808	1.833	0.971	0.939	6.854	7.100
4	1.869	2.618	2.082	2.164	1.871	1.852	1.306	1.013	7.505	7.608
6	3.483	4.053	2.771	2.407	2.210	1.960	1.521	1.174	9.481	9.268
8	3.872	5.224	2.627	2.196	2.137	1.854	1.556	1.292	9.132	10.106
11	4.864	6.387	2.908	2.882	2.454	2.362	2.047	2.006	11.762	14.502
14	6.926	9.453	3.999	3.664	3.167	2.864	2.653	2.554	26.812	29.586

INFLUENCE OF INDOLEACETIC ACID ON TOTAL NITROGEN DISTRIBUTION

In general, the results presented in table 3 indicate that treatment with indoleacetic acid resulted in greater transfer of nitrogen from the upper parts of the seedlings, particularly from the leaves, into the lower hypocotyl and roots. The treated roots and lower hypocotyls are, with few exceptions, higher at each sampling than the roots and lower hypocotyls of the untreated seedlings; however, the differences in total nitrogen between the treated and untreated roots at every stage of sampling except the first is greater in the high nitrogen, low carbohydrate seedlings than in the low nitrogen, high carbohydrate seedlings.

During the first 8–11 days after treatment, the upper hypocotyl, first inter-

nodes, and leaves of the treated, high nitrogen, low carbohydrate seedlings showed greater percentage loss of total nitrogen than did the same parts of untreated seedlings. At 11 or 14 days, however, the percentage of total nitrogen in these parts was higher in the treated seedlings than in the untreated. In the low nitrogen, high carbohydrate seedlings the movement of nitrogen from the upper portions of the treated seedlings occurred to a lesser degree than in the treated, high nitrogen, low carbohydrate seedlings. In fact, in most cases the percentage of nitrogen in the upper hypocotyl and first internodes of the treated, low nitrogen,

TABLE 3

PERCENTAGE TOTAL NITROGEN CONTENT OF KIDNEY BEAN SEEDLINGS TREATED WITH WATER AND WITH 0.01 PER CENT AQUEOUS SOLUTION OF INDOLEACETIC ACID

Days after treatment	Roots		Lower hypocotyl		Upper hypocotyl		First internodes		Leaves	
	Water	Acid	Water	Acid	Water	Acid	Water	Acid	Water	Acid
	High nitrogen, low carbohydrate									
0	5.07	.....	6.72	.....	6.42	.....	6.42	.....	7.62	.....
2	2.72	2.97	6.60	6.17	6.65	6.62	6.25	6.15	7.27	6.75
4	3.22	3.90	6.20	6.20	6.47	6.35	6.35	6.30	6.80	6.20
6	3.17	4.10	6.00	5.90	5.87	5.67	5.75	5.65	6.35	5.62
8	2.55	3.02	5.52	5.82	5.42	5.35	5.55	5.17	6.40	5.85
11	2.87	3.77	4.00	5.00	3.60	4.65	3.82	4.45	5.52	5.10
14	2.32	3.37	3.12	5.50	2.70	4.87	2.75	4.67	4.90	5.32
	Low nitrogen, high carbohydrate									
0	3.27	.....	5.00	.....	4.87	.....	4.70	.....	6.27	.....
2	3.20	3.57	2.85	5.80	5.15	5.67	4.95	4.92	6.85	6.65
4	3.40	4.02	5.10	5.85	4.52	5.52	4.80	4.35	6.85	6.32
6	2.50	3.22	3.70	5.00	3.30	4.67	3.65	4.70	5.82	5.70
8	2.50	2.72	3.15	4.90	2.90	4.52	3.25	4.35	5.22	5.27
11	2.22	1.90	2.32	3.22	2.00	3.02	2.22	2.87	4.35	4.60
14	1.87	2.05	1.45	3.15	1.20	2.40	1.50	2.40	4.50	4.02

high carbohydrate seedlings was higher than that in the same parts of the untreated seedlings. The results indicate that the effect of treatment on the distribution of total nitrogen is affected by the level of nitrogen nutrition.

Influence of indoleacetic acid on distribution of carbohydrates

At almost every period of sampling the total sugar (table 4) is lower in all parts of the treated seedlings than in the same portions of untreated seedlings. One of the probable reasons for the decreased total sugar content of the roots

and lower hypocotyls of the treated seedlings may be that the sugars in these regions were being more rapidly converted into proteins and other insoluble compounds, owing to the increased rate of root development. Likewise, the decreased total sugar in the upper parts of the treated plants may be due to the transfer of sugars out of this region into the lower parts of the seedlings.

TABLE 4

PERCENTAGE TOTAL SUGARS OF KIDNEY BEAN SEEDLINGS TREATED WITH WATER AND WITH 0.01 PER CENT AQUEOUS SOLUTION OF INDOLEACETIC ACID

Days after treatment	Roots		Lower hypocotyl		Upper hypocotyl		First internodes		Leaves	
	Water	Acid	Water	Acid	Water	Acid	Water	Acid	Water	Acid
	High nitrogen, low carbohydrate									
0	None	.....	0.08	.....	0.40	.....	2.07	.....	0.08	.....
2	None	None	0.16	0.12	None	None	None	None	0.04	0.04
4	1.07	0.44	0.24	0.16	None	None	1.90	0.40	0.08	0.56
6	1.63	0.24	3.77	0.24	None	None	2.50	0.95	1.80	0.74
8	1.99	0.80	3.19	0.24	0.72	0.24	3.20	3.98	1.70	1.11
11	1.60	0.48	5.81	1.95	1.23	0.52	6.23	3.24	2.59	1.19
14	1.98	1.75	4.68	0.92	5.22	0.95	5.10	3.71	2.25	1.39
	Low nitrogen, high carbohydrate									
0	0.76	.....	0.64	.....	1.91	.....	5.40	.....	1.01	.....
2	None	None	0.16	0.20	None	None	0.88	0.32	0.04	0.04
4	2.69	1.23	3.16	0.24	None	None	4.30	0.32	0.91	0.16
6	1.91	0.24	5.57	0.28	2.07	0.24	4.76	4.11	4.12	1.39
8	2.15	1.11	2.15	0.24	1.23	0.30	4.33	7.37	1.27	1.17
11	1.19	0.40	3.24	1.63	4.82	4.45	7.84	4.78	2.57	1.80
14	3.06	0.80	5.77	3.02	6.74	4.41	7.84	4.92	2.44	2.03

The lower and upper hypocotyls of treated seedlings of both levels of nutrition showed a greater percentage of alcohol-insoluble acid-hydrolyzable carbohydrates 2 days after treatment than did the same parts of untreated seedlings (table 5). In most other instances, however, after this period the percentage of alcohol-insoluble acid-hydrolyzable carbohydrates was higher in all parts of the untreated seedlings than of the treated, except in the leaves of the low nitrogen, high carbohydrate seedlings. The reason that the percentage of this carbohydrate fraction is usually higher in all parts of the untreated seedlings than in the treated probably is the same as given for the higher total sugars in all parts of the untreated seedlings.

TABLE 5

PERCENTAGE ALCOHOL-INSOLUBLE ACID-HYDROLYZABLE CARBOHYDRATES OF KIDNEY BEAN SEEDLINGS TREATED WITH WATER AND 0.01 PER CENT AQUEOUS SOLUTION OF INDOLEACETIC ACID

Days after treatment	Lower hypocotyl		Upper hypocotyl		First internodes		Leaves	
	Water	Acid	Water	Acid	Water	Acid	Water	Acid
	High nitrogen, low carbohydrate							
0	5.03	.....	8.92	.....	22.10	.....	5.88	.....
2	3.54	4.32	9.91	11.05	17.85	25.77	8.57	4.62
4	11.19	10.20	11.47	10.41	19.68	17.07	9.05	7.84
6	5.03	7.58	12.32	12.11	17.56	18.83	8.64	8.14
8	12.79	13.32	16.14	15.36	16.85	18.76	7.42	7.22
11	19.61	14.99	22.09	13.10	24.42	13.86	10.13	7.65
14	.....	.....	27.75	15.10	21.81	14.58	9.67	7.82
	Low nitrogen, high carbohydrate							
0	8.99	.....	13.88	.....	23.37	.....	9.42	.....
2	3.89	4.11	14.66	16.28	31.01	22.23	6.48	7.12
4	3.82	2.97	13.17	12.11	17.29	27.33	7.42	7.65
6	16.57	9.70	19.26	16.29	21.09	21.58	9.03	6.23
8	20.18	15.10	20.25	15.22	16.55	16.24	7.65	7.26
11	23.08	14.16	26.06	16.85	22.30	21.10	9.37	10.41
14	24.17	15.20	35.82	22.75	24.22	20.04	15.89	10.48

Summary

1. One lot of kidney bean seedlings was grown under conditions of high nitrogen and low carbohydrate; another lot with low nitrogen and high carbohydrate. After harvesting, dry weight, total nitrogen to include nitrates, total sugars, and alcohol-insoluble acid-hydrolyzable carbohydrates determinations were made.

2. Treatment with indoleacetic acid greatly increased the rate of root formation in the low nitrogen, high carbohydrate seedlings. The response of the high nitrogen, low carbohydrate seedlings to treatment was much slower. Apparently the carbohydrates were the limiting factor in the production of new roots after treating the high nitrogen, low carbohydrate seedlings with the acid.

3. The high nitrogen, low carbohydrate seedlings were less responsive to changes in distribution of dry weight. This lack of or slow response to was probably due to the low supply of available carbohydrates which could be translocated to the region of treatment.

4. In general, treatment with indoleacetic acid was accompanied by greater

transfer of nitrogen from the upper part of the seedlings to the lower parts than in the untreated seedlings. The movement of nitrogen following treatment was greater from leaves to roots and lower hypocotyl in the high nitrogen, low carbohydrate seedlings than in those low in nitrogen and high in carbohydrates.

5. Treatment decreased the percentage of total sugars in all parts of the seedlings. The percentage total sugars in all parts of the treated seedlings at both levels of nutrition was still lower at the end of the experiment than in those seedlings not treated with the growth substance.

6. In general, the percentage alcohol-insoluble acid-hydrolyzable carbohydrates increased in the hypocotyl and to a lesser extent in the leaves with the age of the seedling and to a greater degree in untreated plants than in those which had been treated. The percentage of hydrolyzable carbohydrates in the first internode of untreated seedlings was approximately the same at the end of the experiment as at the beginning, whereas internodes of treated seedlings decreased in percentage of this constituent.

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METHOD FOR COVERING EMASCULATED FLOWERS IN PLANT BREEDING

J. R. KING

(WITH FOUR FIGURES)

Plant breeders have always been concerned with the problem of properly covering emasculated blossoms as a protection against contamination by insect visits. Because of the many difficulties experienced with paper bags and similar covers, an effort was made to secure a covering that would eliminate some of these disadvantages. Such a cover should be easy to put over a branch and should be insect proof. It must be resistant to wind and rain and also must permit pollen germination beneath it and air circulation through it.

In the development of an idea for making such a cover, Warner Bros. Pictures co-operated in the spring of 1938 by demonstrating to me and then lending me a "mechanical spider." Later a small and modified model of this machine was built at Davis, California.

The web-spinning machine (fig. 1) works on the principle of centrifugal force. A specially designed centrifuge cup is mounted on the drive shaft of a fractional horse-power electric motor and in front of a fan. Rubber cement is used to make the webs. The cement is forced out by centrifugal force through the two lips of the whirling cup and is driven by a fan in the form of a revolving thin mass, which quickly spins itself into a web wherever it comes into contact with an object.

The web may be made as thick as desired and can be spun over any part or over all of a large branch (fig. 2). It is applied quickly and easily and does not damage the pistils, even when torn off. Pollen will germinate beneath the cements and cement mixtures tested, and the webs are insect proof when carefully and evenly spun (figs. 3, 4).

In the spinning process, the small strands of rubber cement are actually laid over the flower parts, so that there is no detectable damage from abrasive action such as may occur when a bag is put over a branch. There is no stretching or pulling during the spinning. The breeze created by the electric-fan blades will bend many outside pistils so that the rubber strands strike and accumulate on the styles and ovaries while the stigmas are untouched and point away from the web. This gives added protection from insects that may alight on the web. The web may be made more wind-and-rain resistant by quickly wrapping loose or flowing rubber strands around the body of the web or by pressing them against it (fig. 2). As the web is, of course, less easy to remove than a paper bag or similar cover, the more

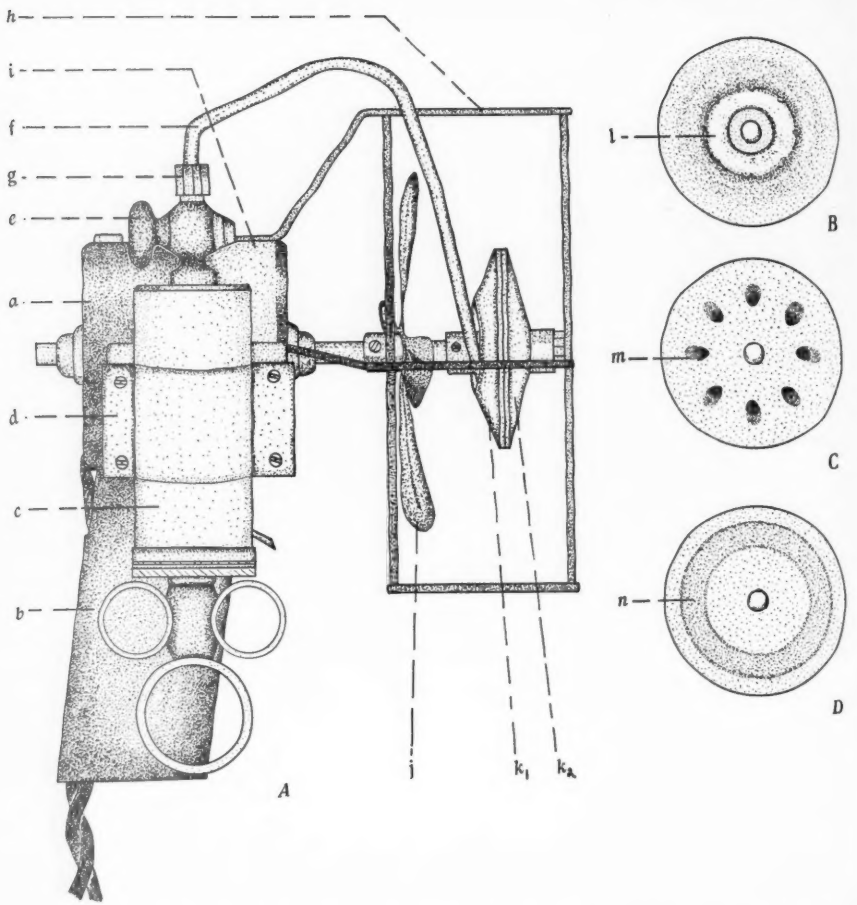
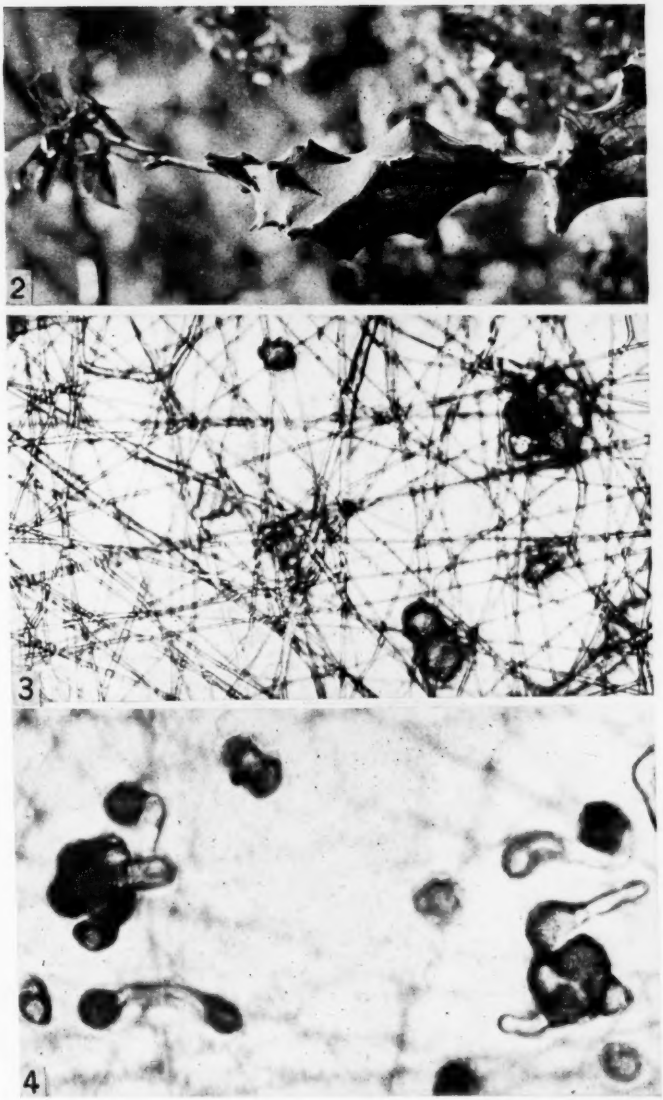


FIG. 1.—*A*, machine used in spinning rubber cement webs over emasculated flowers in plant breeding: (*a*) fractional HP electric motor with pistol grip (*b*); (*c*) metal syringe containing rubber cement; (*d*) metal strap fastening syringe to motor; (*e*) stopcock regulating flow of cement through metal tube (*f*); (g) compression fitting connecting copper tube with stopcock; (*h*) wire guard around fan blades and centrifuge, the frame clamped to the motor by a metal band (*i*); (*j*) fan; (k_1 and k_2) rear and front disks, respectively, of centrifuge. *B*, *C*, outer and inner face views, respectively, of rear centrifuge disk: (*l*) well into which cement flows from copper tube; (*m*) holes through which the cement flows from well in outer face of rear disk into recess of front disk. *D*, inner face view of front centrifuge disk: (*n*) recess in inner face of front disk from which the cement is forced through the lips of the centrifuge.



FIGS. 2–4.—Fig. 2, rubber cement web covering group of emasculated cherry flowers. Fig. 3, moderately heavy web spun over culture of Royal apricot pollen. Note size of pollen grains in relation to size of spaces between the strands of the web. Fig. 4, germination of Royal apricot pollen beneath rubber cement web. Focus of photograph is past the web, hazy outline of which is evident in background.

desirable procedure is to pollinate soon after emasculation, then to spin the web over the branch.

Rubber cements and cement mixtures vary considerably in their ease of spinning, their strength, and their durability under field conditions. The strongest, most durable mixture thus far tested[1] consists of 1 part N-butyl Methacrylate Polymer (dissolved in toluene), 2 parts Goodrich cement No. 1, 2 parts Goodrich cement No. 3, and 4 parts Goodrich cement No. 8540. This mixture is resistant to wind and rain, and has withstood the sun's rays and high temperature for over 10 days. More than sixty rubber cements or cement mixtures have been tested, and improvements in this relation are being sought constantly. Synthetic products have not thus far proved, on the whole, so adaptable to this work as natural rubber.

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[1] I am indebted to The B. F. Goodrich Company for co-operation in working out the many problems relative to the rubber cements.

CURRENT LITERATURE

General Bacteriology. By D. B. SWINGLE. New York: D. Van Nostrand Company, Inc., 1940. Pp. xii+313. Figs. 157. $3.00.

This book is designed to give the general science student a well-rounded knowledge of the subject. It assumes that he has had a fundamental course in biology and also has knowledge of elementary chemistry, including organic chemistry.

The subject matter is well balanced and is arranged logically and concisely. The first ten chapters cover the subjects of historical development of bacteriology, microscope and culture methods, morphology, classification, reproduction and growth, nutrition, bacterial products, and effects of the environment on bacteria. Two chapters are devoted to molds, yeasts, and related fungi. The remaining twelve chapters cover the subjects of activities of bacteria in the soil; bacteriology of water supplies, and water examination; the role of bacteria in sewage disposal; microorganisms of the air; milk and milk products; foods; industrial microbiology; infection and immunity.

While the essential points in this broad range of subject matter are well brought out within a minimum of space, a few suggestions might be made. In the discussion of the growth curve of a culture of bacteria (pp. 90–91), the relationship of the *normal* changes in cell size and morphology to the different parts of the curve could have been brought out more clearly. The statements on the composition of the yeast cell wall on pages 156 and 157 appear contradictory. In the chapter dealing with historical development, the subheadings "Discovery of vaccines" and "Discovery of antitoxic sera" might perhaps have been better termed discovery (or development) of the principles of immunization. Chapter XXI on the mechanism of infection seems rather brief. In the chapter on immunity, the discussion of phagocytosis conveys the impression that the white blood cells are the only kind of phagocytic cell. Some explanation of the macrophage and its important role might well have been included.

On the whole the text is relatively free from the minor errors which so often plague first editions. An exception occurs on page 211, however, where the genus *Alcaligenes* is spelled three different ways. The book is well illustrated and many of the illustrations are original.—S. A. KOSER.

North American Cariceae. By KENNETH KENT MACKENZIE (posthumous). Illustrated by HARRY CHARLES CREUTZBURG and edited by HAROLD WILLIAM RICKETT. New York: New York Botanical Garden, 1940. Vol. I: pp. iv+1–269, pls. 1–269; Vol. II: pp. 270–547, pls. 270–539.

A sumptuous work, containing descriptions in English, also individual full-page plates, of the 533 species of *Carex* and six other species of Cariceae (three of *Kobresia*, two of *Uncinia*, and one of *Cymophyllus*) previously treated by the late K. K. MACKENZIE in his monograph of the Cariceae (North Amer. Flora 18:1–478. 1931–35). Keys and detailed lists of synonyms are omitted, since these are available in the earlier, monographic treatment. Nor are the descriptions as full as in the earlier work, since many characters have been portrayed by the artist, MR. CREUTZBURG.

A brief note on geographic distribution accompanies each species. This is followed by a precise statement of the one or often several sources, in the herbarium, of material on which the illustration was based—a feature one cannot commend too highly.

This great work may well afford pride to its editor, DR. RICKETT, and to the other authorities of the New York Botanical Garden, to which the author had bequeathed his drawings and a fund to provide for their publication.—E. E. SHERFF.

Biological Symposia. Vol. I. Edited by JAQUES CATTELL. Lancaster, Pa.: The Jaques Cattell Press, 1940. Pp. vii+238. Illustrated.

The Richmond meeting of the American Association for the Advancement of Science was particularly fortunate in the biological symposia it offered, and this volume presents the papers given at three of these meetings.

Two sessions were devoted to a discussion of the cell theory—the development of the theory and the techniques which have made knowledge of cells possible. The discussion included the modern concepts of the cell as a structural unit, its behavior in development and reproduction, and a philosophical look into the future. The zoologists held a symposium on mating types and their interaction in the ciliate Infusoria. Recent investigations in this line have opened up entirely new fields of research. At a joint session of zoologists and geneticists the subject of chromosome structure was considered. Technical development during recent years and the application of physics and chemistry to the problems have made great advances in the knowledge of this subject possible.

The three biological symposia form a related series on an important theme, and the editor is to be commended for bringing them together in one volume and thus making them available to a larger interested public.—J. M. BEAL.

Natural History Index-Guide. Compiled by BRENT ALTSHELER. 2d ed., rev. and enl. New York: H. W. Wilson, 1940. Pp. 583. Sold on service basis.

The difficult problem of quickly locating widely scattered articles in books and periodicals dealing with the field of natural history in its broadest definition is rendered simpler by this index. In all there are 54,063 references to the indexed publications, entered under 9477 subject headings. These headings appear in one alphabetical index with references to material in fifteen major sections: astronomy, atmosphere, botany, food and drink, geography, geology, hygiene and health, microscopy, paleontology, zoology, etc. The compiler was assisted by an able list of collaborators in several scientific institutions.—E. J. KRAUS.

The Evolution of the Land Plants (Embryophyta). By DOUGLAS HOUGHTON CAMPBELL. Stanford University, Calif.: Stanford University Press, 1940. Pp. ix+731. Illustrated. $6.50.

The structure and phylogeny of plants commonly included in the phyla Bryophyta, Pteridophyta, and Spermatophyta are discussed in a general and comparative manner in this large volume.

The classification of the Embryophyta and the range of structure of their gametophytes and sporophytes are first discussed, after which the Bryophyta, Psilophyta, Lycopodineae, Equisetineae, and Filicineae are presented. The Salviniaceae and Marsileaceae are included in one chapter although lack of relationship other than heterospory is acknowledged. Heterospory and the seed habit are ably presented in a separate chapter. Fossil plants receive adequate treatment in the respective plant groups. Less than half the book is devoted to the gymnosperms and angiosperms, with a well balanced treatment of the former. Besides introductory discussions of

the flower and other structures, and of pollination and fertilization in the angiosperms in general and the monocotyledons and dicotyledons in particular, the orders and families of the flowering plants are each discussed briefly. Many families, such as Onagraceae, Oleaceae, and Gentianaceae, are given only a short paragraph each, while the Polemoniaceae and Hydrophyllaceae are grouped together and receive only two sentences of information. As a rule, organographic and anatomical information is given for the orders, while under the family headings only the best known genera are listed and the geographical distribution of the family noted. Genera such as *Phylloglossum*, *Matonia*, and *Dipteris* of the "Pteridophyta" receive a more extensive treatment than in any other single compilation.

The many line drawings are grouped to good advantage, but unfortunately some failed to print clearly or perfectly. At times the research of other authors is mentioned in the text but no citation is given in the bibliography.

The book is to be recommended as a source of general information about the plants under consideration and as a textbook for courses covering many genera without minute detail.—P. D. Voth.

The New Illustrated Flora of the Hawaiian Islands (*Flora Hawaiiensis*). By Otto Degener. Honolulu, Terr. Hawaii: the author, 1933–1940. Four centuries, unpaged, illustrated, each $3.50.

This work, of which advance notice was given some years ago in these columns (91:465, 1931), has now been completed through four centuries of plates, with accompanying text. In accordance with the scheme adopted at its inception, its various treatments of both native and introduced plants appear from time to time, on the loose-leaf plan. With the completion of each new century, the subscriber is supplied with an attractive cover and is expected to rearrange all the treatments to date so as to accord with established family sequence. The numerous plates are well executed and mostly from original drawings by artists whom the author has personally trained. Much of the text is based upon the author's own studies or upon recently completed researches of special students of the Hawaiian flora. In this way the author is producing, within the limits of a single work, an increasingly comprehensive and highly authoritative digest of our knowledge of this flora up to the present time.—E. E. Sherff.

The Genus Tulipa. By Sir A. Daniel Hall. London: The Royal Horticultural Society, 1940. Pp. viii+171. Illustrated.

The opening chapters deal with the history and distribution of the genus, followed by chapters on morphology, cytology, and taxonomy. Since polyploidy is common in *Tulipa*, the cytological studies have cleared up much confusion in nomenclature, have eliminated many synonyms, and assisted toward the establishment of natural subdivisions in the genus.

An interesting feature of the book is that it deals only with species in the living state and which have been studied over a period of years in the collection at the John Innes Horticultural Institution. The author holds strongly that only in this way can the species of a genus like *Tulipa* be properly discriminated. On this account the book is described as a review rather than a monograph, since it embraces critical descriptions of about 100 species only—all that have been in cultivation in western Europe. Other species, almost wholly of central Asiatic origin, which the author was unable to obtain and grow, have been included in an appendix, with a brief summary of their characters derived from their original descriptions.

The book is beautifully illustrated with many colored plates as well as line drawings. It is recommended to all interested in this group of attractive ornamental plants.—J. M. Beal.

Flora of Indiana. By CHARLES C. DEAM. Indianapolis, Indiana: Department of Conservation, Division of Forestry, 1940. Pp. 1236. Illustrated. $3.50.

This is an exceptionally detailed and critical treatment of a state flora. Distributional ranges are illustrated by 2243 maps. Special features of particular helpfulness to users are the list of more than 700 excluded species; summary of species, varieties, forms, and hybrids; list of new varieties, forms, and combinations published in the text; list of obsolete or not widely used names of localities; reference list of Indiana collectors; list of habitat and distribution terms used; bibliography; and charts showing average dates of last killing frost in spring and first killing frost in autumn for various localities in Indiana.—E. E. SHERFF.